INFLUENCE OF TUMOR ENVIRONMENT AND HOST IMMUNITY ON TUMOR PROGRESSION AND METASTASIS

By

NANETTE P. PARRATTO

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1988

DEDICATION

This work is dedicated to my father, for his patience and loving support over the many years of its duration; to the memory of my mother who taught me well the power of perseverance; and to the memory my trusting friend, O'Dee, for her unconditional love.

ACKNOWLEDGEMENTS

Dr. Arthur Kimura's gentle and persuasive guidance is most gratefully acknowledged. He has taught me to think like a scientist (we hope). The constructive criticisms of my graduate committee advisors, Dr. Richard T. Smith, Dr. Sigurd Normann, Dr. K. Kendall Pierson, Dr. Maron Calderwood-Mays, and Dr. Chris West, are also greatly appreciated. The invaluable advice of Dr. Byron Croker, Cynthia Bevis, and Norma Houghwout regarding immunohistochemical techniques is much appreciated. The contributions of Roberto Luchetta, Linda Lee-Ambrose, Janice Odelbralski, Xiang-Hong Tian, and Donald Dugger toward the completion of this work is also much appreciated.

TABLE OF CONTENTS

LIST OF TABLES

page

LIST OF FIGURES

KEY TO ABBREVIATIONS

aminoethylcarbazole (AEC)

antibody (Ab)

avidin-biotin-horseradish peroxidase complex (ABC)

counts per minute (cpm)

Eagles Hank's amino acid media (EHAA)

fetal bovine serum (FBS)

fluorescein isothiocyanate (FITC)

fluorescein isothiocyanate conjugated F(ab')2 sheep anti-mouse IgG (FITC-SAM)

flow cytometry (FACS)

Gray (Gy)

intraperitoneally (i.p.)

intravenous (i.v.)

monoclonal antibody (MoAb)

newborn calf serum (NCS)

normal mouse serum (NMS)

phosphate buffered saline (PBS)

phosphate buffered saline with 0.7 mM EDTA and 0.6 mM glucose (cPEG)

protein A (pA)

radioimmunoassay (RIA)

streptavidin (sA)

subcutaneous (s.c.)

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

INFLUENCE OF TUMOR ENVIRONMENT AND HOST IMMUNITY ON TUMOR PROGRESSION AND METASTASIS

By

Nanette P. Parratto

December 1988

Chairman: Arthur K. Kimura
Major Department: Pathology

Monoclonal antibodies (MoAb) against Met-72, a 72,000-dalton cell surface glycoprotein, have been used to characterize B16 melanoma variants expressing high experimental metastatic activity. Freshly isolated bone marrow and ovarian metastases of B16 melanoma were found to express high levels of Met-72 by radioimmunoassay (RIA). Lung and ovarian metastases were shown immunocytochemically to contain melanoma cells with heterogeneous levels of Met-72 antigen. Flow cytometric analysis (FACS) of an ovarian metastasis revealed a discrete subpopulation of positive cells. Clones derived from this ovarian metastasis were found to express high levels of Met-72 and high experimental metastatic potential to ovaries. Finally, Met-72 positive variants were localized by routine immunohistology within developing metastatic and subcutaneous B16 melanomas. The few

cells which showed high intensity Met-72 staining have been consistently observed to be within microscopic foci in colonized organs and positioned within regions of subcutaneous masses.

Melanoma has been shown to be immunogenic in experimental and clinical situations; however, syngeneic antibodies (Ab) against highly metastatic variants have only been obtained by experimental manipulation of the host immune response. Binding of anti-B16 melanoma Ab elicited by syngeneic intravenous immunization was inversely correlated with metastatic activity of melanoma lines and clones by RIA. Flow cytometric analyses similarly revealed that the proportion of reactive cells in melanoma populations was inversely correlated to their experimental metastatic activity. Anti-B16 melanoma MoAb derived by hybridoma technology from intravenously challenged mice, showed distinct anti-melanoma specificity in RIA. Monoclonal antibody directed against non-metastatic melanoma variants defined specific bands in Western blot analyses of Mr 45,000 and 50,000. Finally, negative cellular selection techniques revealed an increased experimental metastatic potential associated with the non-immunogenic B16 melanoma subpopulation.

The results of these experiments suggest that 1) Met-72 antigen expression may be a common surface phenotype of B16 melanoma metastatic variants; 2) microenvironmental factors may be instrumental in the induction, attraction or

maintenance of metastatic variants, and 3) the host antibody response may be a key factor in the regulation of levels of metastatic variants during tumor progression by selectively targeting poorly metastatic populations.

INTRODUCTION AND REVIEW OF LITERATURE

The dynamics of tumor cell heterogeneity is clearly evident from clinical and experimental studies of tumor progression (1,2). The diagnosis and treatment of solid tumors has been seriously impaired by a poor understanding of the clonal evolution of heterogeneous populations within developing tumors (3,4). Experimental evidence documents the existence of subpopulations of metastatic tumor cell variants, exhibiting a range of metastatic potentials from low to high (3-6). The precise nature and expression of the phenotypic characteristics during the natural history of metastasis remains unique to each tumor bearing host and the particular solid tumor (7). Clearly, classical pathologic descriptions have been inadequate to identify unique metastatic variants within surgical specimens. This remains a hindrance to quantitative classification of metastatic potential and patient prognosis.

Paulus et al. (8) have implicated regional variations of differentiation in B16 melanoma, localized by morphologic heterogeneity. Direct, in situ visualization of metastatic tumor cell variants has not been possible until recently. We have identified a Mr 72,000 cell surface glycoprotein (Met-72) quantitatively associated with highly metastatic tumor cell

variants of the B16 melanoma (5). More recently, a Mr 83,000 native form of this surface molecule (Met-72/83) has been described (9). The experimental metastatic potential of over 30 B16 melanoma clones has been correlated to a quantitative surface expression of Met-72 (5,10,11). In addition, fluorescence activated cell sorting (FACS) has now been used to directly isolate metastatic variants from the heterogeneous parental B16-F1 tumor (12).

The present study was designed to visualize, isolate and histologically localize metastatic variants present in fresh B16 melanoma tumor masses. Anti-Met-72 monoclonal antibodies (MoAb) reveal a unique localization of Met-72 positive cells along the invading front of the progressing tumor. These experiments show that anti-Met-72 MoAb previously used to characterize the expression of Met-72 *in vitro*, may also be useful for isolation and localization of highly metastatic variants *in situ* in progressing primary and metastatic B16 melanoma.

Much of the success in defining antigenic systems for melanoma is related to the immunogenicity of this tumor type. Syngeneic and autochthonous antibody (Ab) responses against melanoma have been demonstrated in a number of experimental (13-20) and clinical situations (21-24), and yet in syngeneic combinations, Ab against highly metastatic variants have only been obtained through experimental manipulation of the host immune response (5). Taken together, these results suggest

that the immunodominant melanoma population may in fact be those cells with a low metastatic activity.

The current view that tumors are comprised of heterogeneous subpopulations with widely varying and changing metastatic potentials, has been supported in a number of tumor systems (3,4). Selective host responses against the more poorly metastatic subpopulations could thus be expected to dramatically influence the course of tumor growth, progression, and metastasis.

Experiments described here are focused at characterizing the range of Ab specificities elicited by metastatically distinct cell lines and clones of murine melanoma and the potential impact of such responses on tumor progression and metastasis. Antisera and MoAbs derived in these studies have been used to fractionate melanoma populations and deduce the metastatic potentials of the target population. Negatively selected melanoma cells are shown to have enhanced metastatic activity after removal of the anti-melanoma reactive population. These findings strongly support the conclusion that subpopulations of poorly metastatic melanoma are primary targets of host humoral immunity. The relationship of these findings to other anti-melanoma Ab is discussed.

MATERIALS AND METHODS

Mice

C57BL/6, C3H/HeJ, and Balb/c nu/nu mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the Tumor Biology Unit mouse colony, Department of Pathology, in accordance with the National Institutes of Health Guidelines for the Use of Experimental Animals. Female mice, 8 to 16 weeks of age were used in these studies. C57BL/6 mice were exposed to 500 Gy total body irradiation.

Murine Melanoma Cell Lines

Early passages of the B16-F1, B16-F10 and B16-BL6 derivatives of the C57BL/6 melanoma, B16, were obtained from the Division of Cancer Treatment Tumor Bank (E. G. and G. Mason Research Institute, Worchester, MA) where they had been deposited by Dr. I. J. Fidler. A recently derived syngeneic melanoma, JB/RH, was provided by Dr. Jane Berklehammer (AMC Cancer Research Center, Denver, CO) (25,26) for comparison in our studies. Clones of the B16 melanoma were derived by limiting dilution and micromanipulation (5) from both the poorly metastatic B16-F1 cell line and the *in vitro* selected, highly invasive metastatic variant, B16-BL6 (27). Stocks from early passages of all cell lines and clones were frozen at -70°C and restarted every 8 to 12 weeks to limit the

possibilities of functional and phenotypic drift. All cell lines and clones were maintained in vitro at 37°C in a humidified incubator containing 8% CO_2, by subculturing every 4 days. Monolayers of cells were detached from the petri dishes (Costar #3100, Cambridge, MA) by a 3-minute room temperature incubation with 136 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.7 mM EDTA and 0.6 mM glucose (cPEG) (28). For routine passage, cells were washed and replated at a concentration of 5 X 10^5 cells/10 cm dish in 10 ml media.

The poorly metastatic B16-F1 parental line and highly metastatic B16-F10 line were maintained in Cellgro MEM (Sybron, Washington, D.C.) supplemented to 10% with heat inactivated fetal bovine serum (Grand Island Biologicals [GIBCO], Grand Island, NY), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM pyruvate, 2 mM glutamine and 27 mM $NaHCO_3$. The highly metastatic clone BL6-10, derived from B16-BL6, clones derived by limiting dilution from extrapulmonary metastases of B16-F1, and the poorly metastatic line, JB/RH, were maintained in Hanks' balanced salt solution, minimal essential media amino acids, nonessential amino acids and vitamin solution (GIBCO), and nucleosides (Sigma, St. Louis, MO) (EHAA) supplemented to 10% with newborn calf serum (GIBCO), 100 U/ml penicillin, 100 μg/ml streptomycin, 18 mM $NaHCO_3$ and 0.05 mM β-mercaptoethanol (29).

Other Murine Cells and Cell Lines

A panel of murine cells and cell lines was assembled for specificity analysis of the anti-B16-F1 antisera and MoAb secreting hybridomas captured by the immunization procedures. Syngeneic tumor cell lines included PAK17 and PC8 (methylcholanthrene induced fibrosarcomas), Lewis lung carcinoma, EL-4 T-cell lymphoma, and normal syngeneic lung fibroblasts (provided by Dr. Paul Klein, Department of Pathology, University of Florida). An allogeneic B cell lymphoma, 2PK3 (Balb/c origin) was also obtained from the Department of Pathology, Tumor Bank. These cell lines were maintained in Dulbecco's modified minimum essential media (GIBCO) supplemented to 10% with FBS (GIBCO), 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM pyruvate and 2 mM glutamine (5). An allogeneic UV induced melanoma, K1735 (C3H/HeN origin) (30) was obtained from the Department of Pathology, Tumor Bank and maintained in 10% newborn calf serum supplemented EHAA. The P815 mastocytoma of the DBA/2 strain (provided by Dr. Sigurd Normann, Department of Pathology, University of Florida), was maintained in RPMI (Gibco) supplemented to 10% with FBS plus 100 μg/ml streptomycin, 100 U/ml penicillin. Cells were harvested during log phase of growth for use in radioimmunoassays (RIA). Single cell suspensions of normal C57BL/6 and Balb/c spleen cells were prepared by mechanical disaggregation and lysis of RBC in 17 mM Tris, 144 mM NH_4Cl, pH 7.2.

Monoclonal Antibodies

The anti-B16-F1 monoclonal antibodies (MoAb) derived in these studies were generated as described (5,31). Briefly, spleen cells from mice injected i.v. with 3 X 10^5 B16-F1 cells were fused with BALB/c myeloma SP2/O-Ag14 cells and dispensed under limiting dilution conditions into 96 well plates. Supernatants from growth positive wells were screened by RIA for binding to the poorly metastatic melanoma line, JB/RH. The highest binding hybridomas were subcloned and screened for specificity using JB/RH and BL6-10 cells. Monoclonal antibody isotypes were determined by enzyme linked immunosorbant assay (Southern Biotechnology Associates, Inc., Birmingham, AL). Monoclonal antibodies derived by syngeneic immunization of C57BL/6 mice with B16-F1 melanoma (anti-B16-F1 MoAb; 2D8.3B1 and 2H5.1B1, designating clone and subclone) were both found to IgG_3:kappa. Another syngeneic anti-B16-F1 MoAb (1F11.1E12) was found to be IgG_{2a}:kappa. Anti-Met 72 MoAb were generated by syngeneic immunization of C57BL/6 mice with selected B16 melanoma clones. The specificity and characteristics of these MoAbs have been reported in detail (5,31). Hybridoma cells secreting an isotype identical, negative control MoAb used in this study (anti-sheep red blood cell, N-S.7, IgG3:kappa) were obtained from the American Type Culture Collection.

Affinity Purification of Monoclonal Antibody for Biotin and Fluorescein Isothiocyanate Conjugation

Monoclonal antibodies were affinity purified by fractionation through protein A-Sepharose 4B (Pharmacia,

Piscataway, NJ) (32) and purity checked by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (33). Affinity purified IgG (1 mg/ml) was dialyzed against 100 mM $NaHCO_3$, 200 mM NaCl pH 8.2 and then reacted with 2 mM sulfosuccinimidyl 6-(biotinamido)hexanoate (Pierce Chemical, Rockford, IL) in dimethylformamide (Sigma), at biotin ester:protein ratios of 1:2.5 to 1:10 (weight/weight) for 4 hours at room temperature, in the dark. The reaction was stopped by the addition of 1 M NH_4Cl to a final concentration of 100 mM in the reaction mixture. Unreacted biotin was then removed by exhaustive dialysis against PBS. Biotin-conjugated protein concentrations were determined by optical density at 280 nm (34). Optimal biotin ester/protein ratios used for the conjugation of the various preparations were determined empirically by flow cytometric analysis on cell preparations (35). Briefly, 2 X 10^6 melanoma cells were incubated with biotin conjugated antibody at 4°C for 30 min. in the dark. After 3 washes in PBS supplemented to 2% with FBS, either native or biotin conjugated MoAb were incubated with fluorescein isothiocyanate conjugated F(ab')2 sheep anti-mouse IgG (Cooper Biomedical, Malvern, PA) (FITC-SAM) or FITC conjugated avidin (Vector Laboratories, Burlingame,CA). After 3 washes with PBS, 1 X 10^4 cells were counted and analyzed by flow cytometry (12,35).

Fluorescein isothiocyanate (FITC) conjugation was as described by Goding (36). Briefly, affinity purified MoAb

were dialyzed against 80 mM Na_2CO_3, 200 mM $NaHCO_3$ pH 9.5 and reacted with 2 mg FITC per ml dimethylsulfoxide (Sigma) at a FITC/protein molar ratio of 4. Unreacted FITC was separated by desalting through Sephadex G-25 (Pharmacia).

Flow Cytometry

Cultured cell lines were harvested as described for routine passage and washed twice in ice cold PBS. Aliquots of 10^6 cells were incubated at 4°C for 45 min. with diluted normal or immune serum or with free or conjugated MoAb as described in the results section. After two washes with 100 volume excess of ice cold PBS, labeled cells were counterstained with FITC-SAM for 45 minutes at 4°C. Labeled and stained cells were washed, resuspended at 5 X 10^5 cells per ml and filtered through 41μ nylon mesh (Spectrum, Los Angeles, CA). Flow cytometric analysis was performed with the FACSTAR argon laser (Becton/Dickinson, Oxnard, CA) the photomultiplier voltage for green fluorescence set at 400, the amplifier gain set at log scale and the photodiode for forward scatter set at 0 for sample analyses. Data was collected and analyzed by the Becton/Dickinson Consort 30 computer program (Oxnard, CA) (35).

Subcutaneous Tumor Generation and Spontaneous and Experimental Metastasis Assays

Single cell suspensions of B16-F1, B16-F10 or BL6-10 metastatic variant lines were injected s.c. (2 X 10^6 cells per 0.1 ml 136 mM NaCl, 3 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 [PBS]) into the caudal mid-dorsum of age matched normal or 500

Gy irradiated C57BL/6 mice or into Balb/c nu/nu mice, to generate primary tumor masses. Animals were anesthetized with 50 mg/kg Na pentabarbital i.p. to aseptically excise tumors and associated skin at various intervals of tumor growth; the wounds were then clipped. Spontaneous metastasis formation was assessed by sacrifice and necropsy 21 days post-operatively. Experimental metastasis of all melanoma lines was assessed by sacrifice and necropsy 21 days after lateral tail injection of 3 X 10^5 cells/0.2 ml PBS into age matched syngeneic female mice.

Immunocytochemistry, Immunohistology and Direct Immunofluorescence

Cytospin and impression smear preparations of freshly excised B16 melanoma tumor masses were examined for Met-72 expression by immunoperoxidase staining. Single cell suspensions of tumor masses were generated by teasing tissue to 1 mm^3 pieces followed by an 8 to 10 hour incubation at 4°C in 10 ml/gm tumor weight, EHAA supplemented to 0.1% with collagenase V (Sigma) (37). Viable cells were separated on Ficoll Hypaque (Pharmacia), washed, and endogenous peroxidase activity blocked by 30 min. incubation in PBS supplemented to 5% with heat inactivated FBS and 0.3% with H_2O_2 at 4°C. Cells were labeled in the dark with biotin conjugated MoAb for 30 min. at 4°C, washed and incubated with avidin-biotin-horseradish peroxidase complex ([ABC] Vector Laboratories, Burlingame, CA) for 30 min. at 4°C. Approximately 3 X 10^4 labeled cells were cytocentrifuged onto poly-D-lysine coated

microslides, air dried, fixed in acetone for 30 seconds and rehydrated in PBS. The reaction was developed with aminoethylcarbazole ([AEC] Sigma) in the presence of H_2O_2. The slides were washed and counterstained with Harris' hematoxylin (Sigma) (38,39).

Freshly excised tumor masses, rinsed in ice cold PBS, were gently touched to coated slides to create impression smears. Slides were air dried, fixed in acetone for 10 min., rehydrated with PBS and either immediately processed by immunohistology or air dried and stored at -70°C for future use. Impression smears provide a rapid screening tool for detection of antigen expression in vivo (40,41).

B16 melanoma tumors from subcutaneous or experimental metastatic lesions were excised, snap frozen in isopentane chilled with liquid nitrogen and stored at -70°C until sectioned. Subcutaneous masses were examined after 5 to 21 days of growth; metastatic lesions after 14 to 21 days of growth. Skin wounds created surgically on the mid-dorsum were excised at various points during healing, snap frozen and stored at -70°C. C57BL/6 embryos were collected at days 11 and 15 of gestation, snap frozen and stored at -70°C.

Cryostat sections 2 to 12 μm thick were stained according to a modification of described techniques (42,43). Briefly, air dried, acetone fixed, sections were flushed with 0.01% avidin (Vector Laboratories) for 15 min. and then 0.134 mM biotin (Sigma) for 15 min. with a PBS wash between. Sections

were then blocked for endogenous peroxidase activity as for cytospin preparations. Biotin conjugated MoAb at predetermined optimal concentrations were layered onto sections and allowed to react for 30 min. in a dark, humidified chamber. Slides were washed with 20 volume excess PBS and reacted with the ABC reagent for 30 min. The reaction was developed with AEC, the slides counterstained with hematoxylin and examined under oil immersion.

Frozen sections were similarly air dried, acetone fixed and rehydrated with PBS for direct immunofluorescence using FITC conjugated MoAb. Slides were washed with PBS, coverslipped and examined under high power using a 490 nm excitation filter and 510 nm emission filter for green fluorescence.

Immunization Protocols

Age matched female mice were injected via the tail vein with *in vitro* maintained melanoma lines after the cells had been lifted with cPEG and washed three times with ice cold PBS. Mice (4 to 6 per experimental group) were immunized with various numbers of cells and immune sera was harvested at various times to determine dose response and kinetics curves. Optimal dosages and response times were determined and used to further characterize the serologic immune response. Normal mouse serum (NMS) harvested from age matched littermates served as background control. Following exsanguination, animals were killed by cervical dislocation and then

necropsied. Systemic experimental metastatic colonization was assessed by counting lung and extrapulmonary tumor nodules after fixation of the organs in buffered formalin (6).

Radioimmunologic Determination of MoAb and Immune Sera Binding Indices

MoAb binding to the various cell types was measured 1) directly using radiolabeled MoAb (44), or 2) indirectly with ^{125}I labeled protein A as described (45) or with ^{125}I labeled streptavidin (Zymed Laboratories, Inc., San Francisco, CA) for biotin conjugated MoAb using a modification of Philpott, et al. (46). Briefly, 2 X 10^5 freshly harvested cultured cells were incubated for 45 minutes at 4°C with equivalent dilutions of normal or immune mouse serum or MoAb. Washed cells were incubated for an additional 45 minutes with 2 X 10^5 cpm radiolabeled protein A or streptavidin. Radioactivity remaining bound to the cells after washing was assessed by gamma scintillation counting in an LKB gamma counter (LKB Instruments Inc., Gaithersburg, MD). Specific MoAb or immune sera binding to the different cell types is expressed as mean ± S.D. cpm bound or as a binding index (47), calculated from duplicate determinations as follows:

$$\text{Binding index} = \frac{\text{mean cpm bound with specific MoAb or immune sera}}{\text{mean cpm bound with control MoAb or NMS}}$$

This normalizes individual cell line differences in background binding and allows comparison between the different cell types.

Immunomagnetic Bead Separation of Immunoreactive Subpopulations

Immunomagnetic beads (Dynabeads M-450, Dynal Inc., Fort Lee, N.J.) coated with sheep anti-mouse IgG were used to separate cells which had been incubated with normal versus anti-B16-F1 immune sera or anti-sheep RBC MoAb versus anti-B16-F1 MoAb. Cells were harvested from culture, washed and resuspended in hybridoma exhaustion supernatant or PBS supplemented to 5% with normal or immune serum, and incubated for 45 minutes on ice. Immune labeled cells were washed and then incubated with immuno-magnetic beads at a bead to target cell ratio of 3 to 1 for 10 minutes at 4°C. Cells bound to beads were separated from those cells remaining free in suspension by applying a cobalt steel magnetic force (Dynal MPC-1, Dynal Inc., Fort Lee, N. J.) for 1 minute (48). This process was repeated once again with the non-bound cells. The remaining negatively fractionated cells were analyzed by flow cytometry. A portion was injected intravenously at 3 X 10^5 cells per 0.2 ml PBS to assess experimental metastatic capacity.

Cell Solubilization, Polyacrylamide Gel Electrophoresis and Western Blotting

Cell lines maintained in vitro were lifted with cPEG, washed in PBS and solubilized in PBS supplemented to 0.5% with Nonidet P-40 [(NP-40) Bethesda Research Laboratories,

Gaithersburg, MD], 1 % epsilon amino caproic acid and 1 mM phenylmethylsulfonylfluoride (Sigma Chemical Co., St. Louis, MO) at 4°C for 30 min. Supernate was recovered by centrifugation of the detergent lysate for 15 min. at 12,000 X g, 4°C (49,50). Lysates and marker proteins (Rainbow Markers, Amersham, Arlington Heights, IL) were electrophoresed on 5 to 15% or 10% acrylamide slab gels under reducing conditions (33). Proteins were electro-blotted to polyvinylidene difluoride solid membrane (Immobilon, Millipore Corp., Bedford, MA) as described (51,52) and immuno-stained with anti-B16-F1 antisera or anti-B16-F1 MoAb. Antibody bound protein was visualized by protein A conjugated peroxidase catalysis of H_2O_2 in the presence of diaminobenzidene (39).

Statistical Analyses

Significance was determined by analysis of variance, computed using the SAS program (Biostatistics, J. Hillis Miller Health Center, University of Florida).

RESULTS

Anti-Met-72 Monoclonal Antibody Binding to Fixed Melanoma Cells

The highly metastatic B16 melanoma variant, BL6-10, or syngeneic poorly metastatic line, JB/RH (Table 1), were reacted against radiolabeled anti-Met-72 MoAb. Radioactivity remaining bound to fresh or 95% ethanol fixed cells in suspension is shown in Figure 1. The highly metastatic clone, BL6-10, binds equivalently high levels of Ab whether fresh or fixed. Fixation of the poorly metastatic line JB/RH does not artificially increase binding of anti-Met-72 MoAb.

Optimum Biotin Substitution of Monoclonal Antibody

Biotin conjugation to proteins is an empiric reactiion, the optimum conditions varying uniquely for each protein (34). Affinity purified anti-Met-72 or control MoAb were reacted with the biotin ester at varying weight/weight ratios. Flow cytometric analysis of the binding of three different preparations to BL6-10 is shown in Figure 2. The positive fluorescence under curve C in Figure 2 B is comparable to the binding of affinity purified MoAb in Figure 2 A. This ratio of 8 to 1 (protein to biotin) was also found to be optimal for maintenance of control MoAb negative fluorescence.

Biotin Conjugated Anti-Met-72 Monoclonal Antibody Specificity

Reactivity of biotin conjugated MoAb against the highly metastatic B16 melanoma clone, BL6-10, is compared in Figure 3 to the poorly metastatic parental B16-F1 melanoma and to a syngeneic, non-metastatic melanoma, JB/RH (Table 1). There is approximately a five-fold lower level of anti-Met-72 MoAb binding by RIA to the poorly metastatic cell lines. This result documents a retention of specificity in binding to the highly metastatic clone after biotin conjugation.

Fluorescence Activated Cell Sorting Selection of Met-72 Positive Variants Isolated From a Fresh Experimental Ovarian Metastasis

Experimental metastases from the parent B16-F1 melanoma were generated as described. An ovarian metastasis was noted after 21 days and removed for analysis of Met-72 expression by flow cytometry. Biotin conjugated anti-Met-72 MoAb revealed two distinct populations within the freshly excised ovarian metastasis, one highly Met-72 positive (Figure 4). Melanoma cells sorted from this population and maintained *in vitro* have retained a high binding profile to anti-Met-72 MoAb (data not shown). Cells obtained from the original ovarian metastasis (O-1) were cultured, tested by RIA for Met-72 expression (Figure 5, selection cycle 1), cycled *in vivo* to generate experimental metastases and cloned by limiting dilution to obtain O-1.1 (Figure 5). Clone O-1.1 from the original ovarian metastasis was tested for binding to anti-Met-72 MoAb (Figure 5, selection cycle 2) and 3 X 10^5 cells were injected

i.v. in the syngeneic host, resulting in the organ selectivity indicated in Figure 5, selection cycle 2. Subsequent ovarian metastases were isolated, cultured, tested for Met-72 expression and cycled in vivo (Figure 5, O-1.2, cycle 3 and O-1.3, cycle 4). Successive cycles of in vivo passage and culturing were seen to enhance organ selectivity for ovaries (53%, 56%, 87% of the mice having ovarian metastases) and to enrich for Met-72 expression (28, 36, 45 times background) (Figure 5).

Bone Marrow Derived Clones of B16 Melanoma Express High Levels of Met-72

Experimentally derived bone marrow metastases of B16-F1 melanoma were aspirated and mechanically disaggregated into a single cell suspension. Aspirates from five animals were cloned by limiting dilution into 96 well plates. Growth positive wells were expanded in culture and tested for binding to anti-Met-72 MoAb. Clones of bone marrow metastases from each of the five animals are shown in Figure 6, compared to BL6-10 and the parental B16-F1 cells. As seen with the ovarian metastases, Met-72 expression of clones derived from bone marrow metastases was increased above the parental B16-F1 level of expression, and in some cases approached that of the highly metastatic BL6-10 clone.

Immunocytology of Fresh Ovarian and Lung Metastases

B16 melanoma metastatic variants were isolated from fresh lung and ovarian experimental metastases by mechanical and enzymatic disaggregation. Single cell suspensions were

processed for immunocytology using anti-Met-72 MoAb. Cytospin preparations of an experimental ovarian metastasis showed positive immunoperoxidase staining (Figure 7 B). There is cytomorphologic heterogeneity in the level of expression of Met-72 within this mixed cell population. Impression smears of experimental and spontaneous lung metastases generated by injection of the poorly metastatic F1 parental B16 melanoma demonstrated similar levels of individual cellular binding to anti-Met-72 MoAb (Figure 7 D, F). Isotype identical MoAb (anti-sheep RBC, N-S.7) showed no staining in these assays (Figure 7 A, C, E).

Localized Distribution of Met-72 Positive Variants Within Progressing Subcutaneous Melanoma

Immunohistologic analyses of cryostat sections from s.c. B16-F1 parental and BL6-10 melanoma were performed at various time points during tumor growth. Predetermined optimal concentrations of biotin conjugated MoAb were incubated with serial sections of snap frozen tumor. Reactive sites were detected as red granules upon development with AEC. A common pattern of reactivity has been noted in all sections examined from tumors as early as 3 days to as late as 15 days of growth. A subcutaneous B16-F1 derived tumor of 7 days growth serially sectioned and stained with anti-Met-72 MoAb is shown in Figure 8. Background levels of peroxidase staining are shown in Figure 8 A and B, using an isotype identical biotinylated control MoAb, N-S.7. In contrast, Met-72 positive cells were observed to be localized within regions of

the tumor mass (Figure 8 C, D, F). The most brightly Met-72 positive cells were seen within satellites which appear to advance from the main tumor mass. Morphologically, these cells are variably melanotic. Early observations showed a red reaction product around vascular areas (Figure 8 E). A definitive association of this Met-72 positive staining with melanoma cells has not been reproducible. Notably, no binding was seen in the necrotic areas of the tumor mass.

Met-72 Positive Variants Localized Within B16 Melanoma Metastases

Direct immunofluorescence utilizing FITC-conjugated anti-Met-72 MoAb binding on frozen sections of metastases have revealed Met-72 positive variants to be distributed as single cells and micrometastases within colonized organs. These poorly differentiated cells show a predominantly intense cytoplasmic pattern of staining and weaker cell membrane fluorescence (Figure 9 B, E). Diffusely distributed micrometastatic foci of melanotic cells demonstrate a weaker cytoplasmic fluorescent signal (Figure 9 C). Single cells and micrometastases have been observed in lungs colonized with B16-F1 or B16-F10 metastatic variants and in liver colonized with B16-F1 cells (Figure 9 E, F and G).

Specificity Characteristics of Syngeneic Antibodies Generated Against B16 Melanoma

The kinetics of an IgG response against the poorly metastatic B16-F1 or JB/RH lines could be measured as early as 8 days post immunization (Table 2), with moderately higher

binding activity at 16 days (Table 3). In most cases, increasing the immunizing dose above 10^5 cells had little effect on the levels of Ab produced. Antibody responses generated against either of the poorly metastatic lines (B16-F1 and JB/RH) cross-reacted with the other, while the highly metastatic clone was consistently negative in provoking an Ab response or in binding the Ab elicited by the other melanoma lines (Tables 2 and 3).

The ease and reproducibility of this anti-B16-F1 response prompted us to capture these reactivities by hybridoma technology, to further characterize the target cells and surface structures. Using established protocols (5,31), several MoAbs were obtained which demonstrated binding patterns in RIA very similar to anti-B16-F1 antisera. The population distribution of reactive cells was further analyzed by FACS. Specifically, anti-B16-F1 Abs were reacted against selected variants of C57BL/6 melanomas. The results of 6 separate FACS experiments are typified by the example presented in Figure 10 A (antisera) and Figure 10 B (MoAb), where Ab binding (green fluorescence) is plotted versus relative cell number. Antibody binding of anti-B16-F1 antisera or MoAb (stippled) was compared with equivalent dilutions of NMS or control N-S.7 MoAb (solid line). The pattern of reactivity against the immunizing population (B16-F1) is shown in Figure 10 A and 10 B, top left panels. A large proportion of the population shows greater fluorescence

than the NMS. In Figure 10 A, bottom left panel, the high lung colonizing line B16-F10 displayed a qualitatively similar binding pattern, the only difference being a much greater proportion of the cells were coincident with the negative fluorescent population than that seen with the poorly metastatic B16-F1. These differences were not apparent with anti-B16-F1 MoAb (Figure 10 B, bottom left). When our most highly metastatic clone, BL6-10, was subjected to a similar analysis, only marginal reactivity with the anti-B16-F1 Ab was observed (Figure 10 A and 10 B, top right panels). Finally, a recently derived C57BL/6 melanoma, JB/RH, which shows no experimental metastatic activity displayed the highest reactivity with the syngeneic anti-B16-F1 Ab (Figure 10 A and 10 B, bottom right panels).

The second characteristic of Abs generated by these immunizations was the apparent melanoma specificity. As shown in Figure 11, anti-Bl6-F1 MoAbs reacted strongly with syngeneic, poorly metastatic melanomas (Bl6-F1 and JB/RH), moderately with an allogeneic melanoma K1735, and were non-reactive with the other cells types in our tumor panel (fibrosarcoma, lymphomas, carcinoma, mastocytoma) or non-transformed murine fibroblasts or spleen cells.

The third and most important characteristic of anti-B16-F1 Ab was the apparent inverse relationship between Ab binding and experimental metastatic potential of the cell lines and clones tested (Figure 11). This was first suggested by the

reciprocal binding pattern of an anti-B16-F1 MoAb (2D8.3B1) versus our anti-metastatic, Met-72 MoAbs (5) (Figure 11).

Syngeneic Antibody Response Against a C3H Melanoma

The binding of anti-B16-F1 MoAb against a moderately metastatic allogeneic melanoma line, K1735 (30,53), prompted us to examine the specificity of the Ab response to this C3H syngeneic melanoma. Experimental metastases of K1735 were generated and immune sera collected at necropsy. Reactivity against the immunizing line or allogeneic melanoma variants is shown in Figure 12 A. Similar to the Ab response against B16-F1, this response syngeneic to a C3H, moderately metastatic melanoma line was seen to bind higher to the allogeneic, poorly metastatic variants. In addition, the reciprocal binding of anti-B16-F1 versus anti-Met-72 MoAb is apparent on the low versus high metastatic variants, respectively (Figure 12 B).

Metastatic Potential of Melanoma Populations Negatively Selected By Syngeneic Anti-Melanoma Antibodies

Strategies and methodology using immunomagnetic bead cellular affinity separation were developed to negatively select for the non-reactive cells in the heterogeneous immunizing B16-F1 population. Flow cytometric analyses of B16-F1 cells reacted against control (solid line) or immune Ab (stippled line) are shown in Figure 13. Anti-B16-F1 antisera (Figure 13 A) or MoAb (Figure 13 B) binding is compared to non-reactive populations, obtained after two cycles of immunomagnetic bead separation, restained with anti-B16-F1

antisera (Figure 13 C, F) or anti-B16-F1 MoAb (Figure 13 D, E). The efficiency of the immunomagnetic bead separation protocol is apparent in panels C, D, and E.

The FACS profiles of B16-F1 and B16-F10 cells before and after application of the immunomagnetic bead separation procedure are shown in Figure 14. Negatively selected cells obtained by anti-B16-F1 depletion were compared with cells similarly fractionated with NMS or control N-S.7 MoAb. A portion of cells from each of these separations was immediately tested for experimental metastatic activity. As shown in Figure 15, the experimental metastatic potential of control Ab and syngeneic anti-B16-F1 Ab fractionated cells was distinctly different. These results are in agreement with the anticipated doubling in metastatic activity since approximately 60% of the melanoma population was removed by specific Ab + immunomagnetic bead separation. Negligible and significant differences in pulmonary colonization were observed between control and anti-B16-F1 Ab treated B16-F1 and B16-F10 populations, respectively (Table 4). In addition, the fractionation procedure was seen to impact significantly on the extent of extrapulmonary metastases, morbidity, and mortality when compared to controls. As seen in Figure 15, there is a clear trend toward more aggressive extrapulmonary colonization with both the B16-F1 and B16-F10 negatively selected populations. In particular, the involvement of lymph nodes was statistically significant, by analysis of variance,

in the cases of B16-F10 cells negatively selected with antisera (p=0.0196) and MoAb (p=0.0103) (Table 4).

Biochemical Characterization of Anti-B16-F1 Reactive Species

Western blot analysis comparing antisera and MoAb reactivity against detergent lysates of the poorly metastatic lines B16-F1 and JB/RH, versus the highly metastatic B16 clone, BL6-10, is shown in Figure 16. The anti-B16-F1 antisera immuno-stains two distinct bands of Mr 26,000 and 45,000 and a diffuse region between Mr 69,000 to 200,000 with both of the poorly metastatic melanoma lines, B16-F1 and JB/RH (Figure 16 lane b). Two of the anti-B16-F1 MoAb, 2D8.3B1 and 1F11.1E12, identify a corresponding Mr 45,000 band in lysates from the poorly metastatic melanoma lines (Figure 16 lane c and d). A less intensely stained band of Mr 45,000 has been identified using the same anti-B16-F1 MoAb by Western blot analysis of B16-F10 and K1735 detergent lysates (data not shown). In addition, a Mr 50,000 band was stained with the same anti-B16-F1 MoAb in the JB/RH lysate. As anticipated from cell binding assays of the syngeneic antisera, the highly metastatic clone BL6-10 did not reveal any specific bands (Figure 16, lanes b, c, d).

In Situ Characterization of Metastatic Variants Within Experimental Metastases and Subcutaneous Masses of B16 Melanoma

The cellular morphology of metastatic variants expressing Met-72 or anti-B16-F1 antigen was evaluated by immunocytochemical analysis of touch preparations of freshly

excised metastases. Impression smears of liver, lymph node and lung metastases derived experimentally from B16-F1 and BL6-10 cells were examined. A lymph node metastasis derived experimentally by injection of BL6-10 is shown in Figure 17. Variably melanotic cells stained most positively with anti-Met-72 MoAb (Figure 17 B) with individual cellular heterogeneity evident for anti-Met-72 MoAb and anti-B16-F1 MoAb (Figure 17 C) binding. Most cells exfoliated from BL6-10 derived metastases demonstrated background levels of anti-B16-F1 MoAb binding with an occasional (less than 1%) strongly positive cell.

Previously, analyses of cytospin preparations of B16 melanoma ovarian metastases had revealed cellular heterogeneity in Met-72 expression (54). Analysis of frozen sections of experimental metastases revealed similar levels of cytoplasmic and membrane heterogeneity within metastases to the ovary (Figure 18 B) and greater omentum (not shown) when stained using anti-Met-72 MoAb. Anti-B6-F1 MoAb generally showed less intense staining, with an occasional brightly positive cell (less than 1%). Frozen sections of lungs were analyzed using the immunoperoxidase technique for comparison with the earlier direct immunofluorescence studies. Again, Met-72 positive cells were variably melanotic and stained brightly as individual cells or microscopic foci within the parenchyma. The majority of melanotic cells within larger metastatic foci showed weak cytoplasmic staining (Figure 19 C,

D). Interestingly, anti-B16-F1 MoAb revealed strong cytoplasmic and membrane staining within the larger pulmonary metastatic foci (Figure 19 E, F).

To address the effect of the host immune response on the emergence of metastatic variants, B16 melanoma variants were localized within developing s.c. tumors maintained in normal or sublethally irradiated hosts. B16-F1 or BL6-10 cells were injected s.c. into normal or 500 Gy irradiated syngeneic mice and into Balb/c nu/nu mice (2 mice per group). Resulting masses were removed after 12 days, measured in three dimensions to calculate a mean tumor volume (Table 5) and snap frozen. Serial cryostat sections (6 to 12 μm) of each mass from each animal were examined by ABC immunoperoxidase analysis as described. Monoclonal antibody binding was assessed on a scale of negative (lowest) to 5+ (highest) and is summarized in Table 5. Anti-Met-72 MoAb binding was clearly evident within regions of tumors and on variably melanotic cells within normal syngeneic hosts (Figure 20 C and D) as had been noted previously (54). Anti-B16-F1 MoAb binding to serial sections of the same masses clearly showed no reaction within the regions which were most brightly Met-72 positive (Figure 20 E and F). The most striking observation was the increase in the number of Met-72 positive cells and the increase in the level of anti-Met-72 MoAb binding per cell throughout the masses in 500 Gy irradiated syngeneic hosts (Figure 21 C, D and G). This increase was much more apparent

in tumors induced by BL6-10 cells than in those induced by B16-F1 cells. This difference was also more apparent in sublethally irradiated, syngeneic hosts than in allogeneic athymic hosts (Figure 22 C and D). There was a slight increase in the level of anti-B16-F1 MoAb binding per cell within tumors induced by BL6-10 cells maintained in irradiated syngeneic or allogeneic athymic hosts (Table 5, Figures 21 and 22, E and F).

Immunohistology of Syngeneic Healing Skin Wounds and Embryos

Surgically created skin wounds of C57BL/6 mice were examined by ABC immunoperoxidase analysis of serial cryostat sections (6 μm) during early, mid, and late phases of healing. Anti-Met-72 MoAb reactivity was not detected in 24, 48, 72, or 96 hour healing wounds (data not shown).

Embryos of C57BL/6 mice were removed at day 11 and 15 of gestation and snap frozen. Immunohistology of serial cryostat sections (12 μm) were examined from 2 embryos at each time point. Neither anti-Met-72 MoAb nor anti-B16-F1 MoAb (2D8.3B1) binding was detected at either time point during gestation (data not shown).

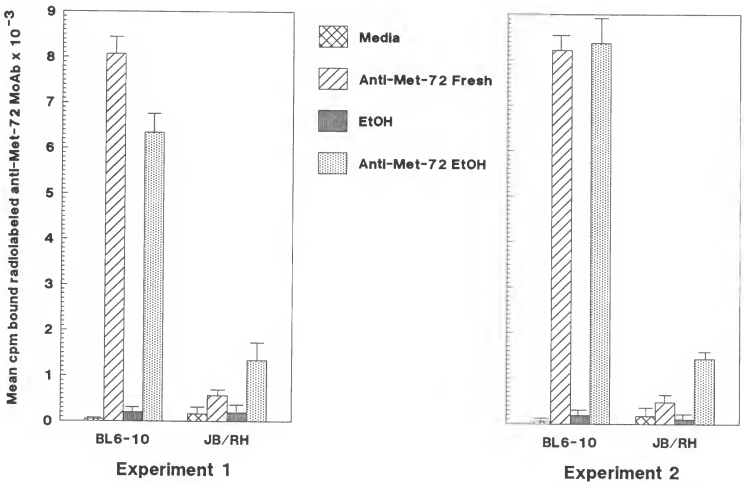
Table 1. Results of Lung Colonization Assays

Cell Inoculum	n[a]	mean # pulmonary nodules ± S.E.	Number of animals with metastases to: Lymph nodes	Ovaries	Bone Marrow	Liver	Kidney
B16-F1	8	15 ± 9	7	1	4	1	1
B16-F1	9	10 ± 12	9	3	3	1	0
B16-F10	8	194 ± 125	1	1	1	0	0
B16-F10	5	113 ± 45	5	3	3	0	1
BL6-10	6	266 ± 87	6	0	3	0	0
BL6-10	5	238 ± 27	5	5	2	0	0
JB/RH	6	0	0	0	0	0	0

a. Number of age matched female mice injected i.v. with 3×10^5 cells per 0.2 ml PBS. Complete necropsies were performed 21 days after tumor cell injection.

Figure 1. Radiolabeled anti-Met-72 monoclonal antibody binding to fresh or ethanol fixed C57BL/6 melanoma cells.

Syngeneic melanoma cell lines were incubated with PBS alone or 95% ethanol at 4°C for 10 min. in suspension, washed with PBS 3 times, and then incubated with ^{125}I labeled anti-Met-72 MoAb for 45 min. at 4°C. Mean ± S.D. cpm remaining bound after 3 additional washes were calculated from four replicate wells in Experiment 1 and six replicate wells in Experiment 2.



Experiment 1

Experiment 2

Figure 2. Optimum biotin conjugation of anti-Met-72 monoclonal antibody.

Affinity purified anti-Met-72 MoAb binding to BL6-10 cells analyzed by FACS indirectly using FITC-SAM (panel A). Biotin was reacted with affinity purified anti-Met-72 MoAb at three biotin ester to protein ratios (A = 1 to 2.5, B = 1 to 5, C = 1 to 8). The binding of these preparations, indirectly indicated by FITC-avidin, is shown in panel B.

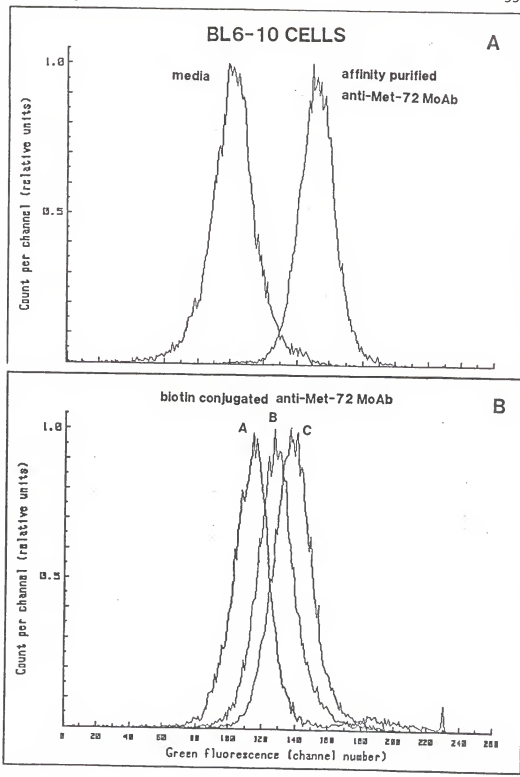

BL6-10 CELLS
A
media
affinity purified
anti-Met-72 MoAb
Count per channel (relative units)
1.0
0.5
biotin conjugated anti-Met-72 MoAb
B
A
B
C
Green fluorescence (channel number)

Figure 3. Retention of binding specificity of anti-Met-72 MoAb after biotin conjugation.

Various amounts of biotin conjugated anti-Met-72 MoAb were reacted with the highly metastatic Bl6 melanoma clone, BL6/l0, the poorly metastatic Bl6 melanoma parent, Bl6-Fl, and a recently derived, poorly metastatic C57BL/6 melanoma, JB/RH. The extent of specific binding was measured after the addition of ^{125}I labeled streptavidin. Values are expressed as the mean of triplicate determinations of cpm bound ± S.D.

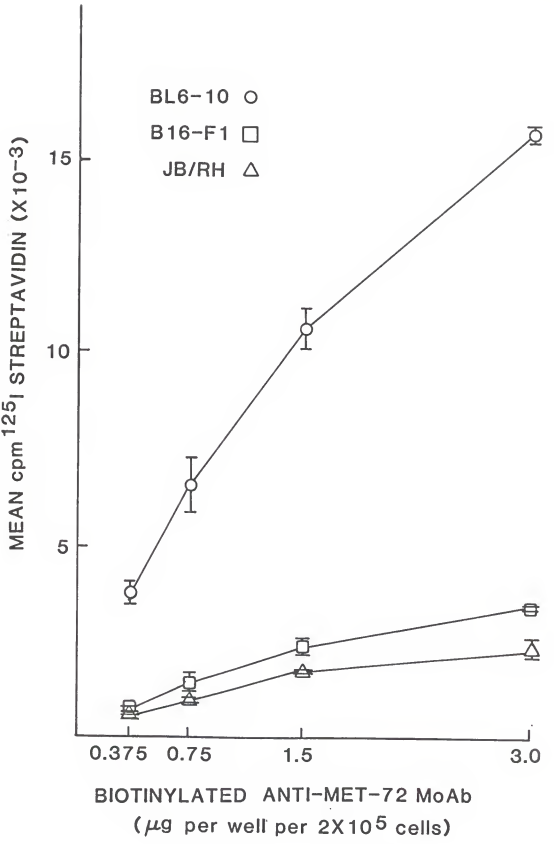

BL6-10 ○
B16-F1 □
JB/RH △
MEAN cpm ^{125}I STREPTAVIDIN (X10^{-3})
15
10
5
0.375
0.75
1.5
3.0
BIOTINYLATED ANTI-MET-72 MoAb
(μg per well per 2X10^{5} cells)

Figure 4. Flow cytometric analysis of Met-72 positive metastatic variants within a fresh ovarian metastasis of B16-F1.

An experimentally derived ovarian metastasis of B16-F1 was mechanically and enzymatically dissociated into a single cell suspension. Cells were reacted with biotin conjugated anti-Met-72 MoAb, washed and counter stained with FITC-avidin for FACS analysis.

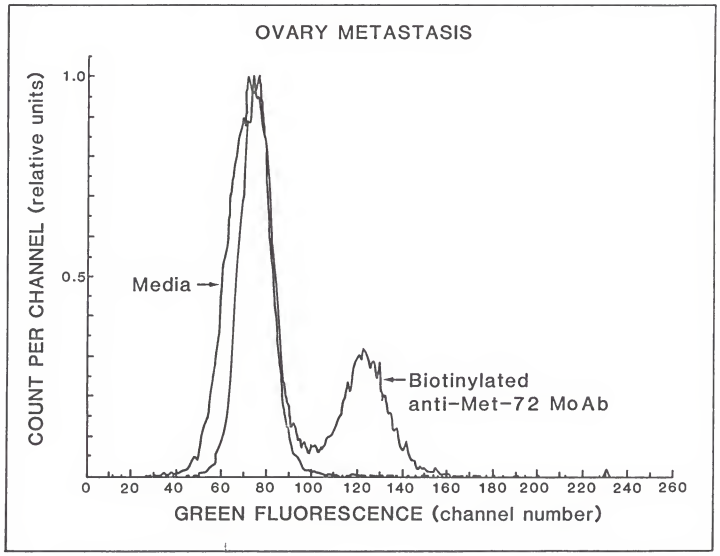

OVARY METASTASIS
COUNT PER CHANNEL (relative units)
1.0
0.5
Media
Biotinylated
anti-Met-72 MoAb
0 20 40 60 80 100 120 140 160 180 200 220 240 260
GREEN FLUORESCENCE (channel number)

Figure 5. Clones derived from experimental metastases to ovaries retain a high expression of Met-72 upon repeated cycling *in vivo*.

a. *in vivo* cycle number for the selection of metastatic variants with ovary specificity.

b. number of mice per group.

c. number of mice with metastases to organ site.

d. Binding of anti-Met-72 MoAb to ovarian metastatic variants as detected by RIA. Results are expressed as a binding index, which is calculated by dividing the mean cpm ^{125}I pA bound with anti-Met-72 MoAb divided by the mean cpm ^{125}I pA bound with N-S.7 MoAb (background binding).

e. Other = kidney, mesentery.

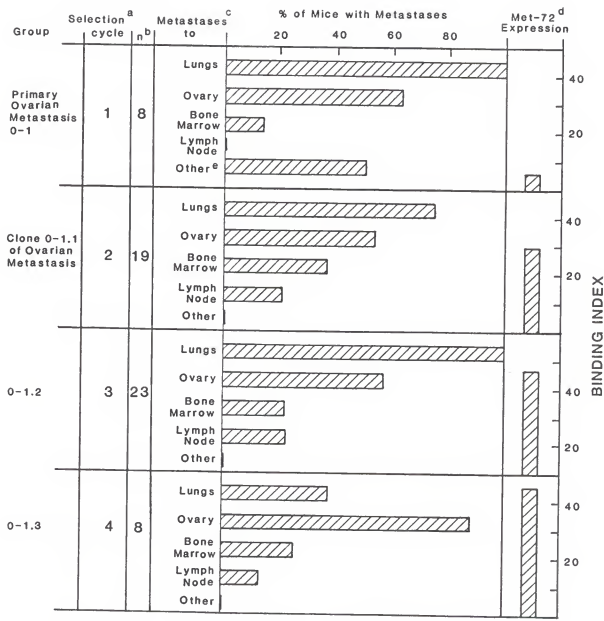

Group
Selection cycle[a]
n[b]
Metastases to[c]
% of Mice with Metastases
20
40
60
80
Met-72[d] Expression
Primary Ovarian Metastasis 0-1
1
8
Clone 0-1.1 of Ovarian Metastasis
2
19
0-1.2
3
23
0-1.3
4
8
Lungs
Ovary
Bone Marrow
Lymph Node
Other[e]
Other
40
20
BINDING INDEX

Figure 6. Anti-Met-72 monoclonal antibody binding to clones of bone marrow derived melanoma metastases.

B16-F1 cells (3×10^5 per 0.2 ml PBS) were injected i.v. and animals were necropsied 21 days later. Bone marrow metastases aspirated from five different animals were cloned by limiting dilution. Five clones (one from each animal) were tested by RIA for Met-72 expression compared to BL6-10 and B16-F1 cells. Values are expressed as the mean of duplicate determinations of cpm bound ± S.D. Background binding of N-S.7 MoAb was routinely less than 600 ± 10%. Data shown is one of seven representative experiments.

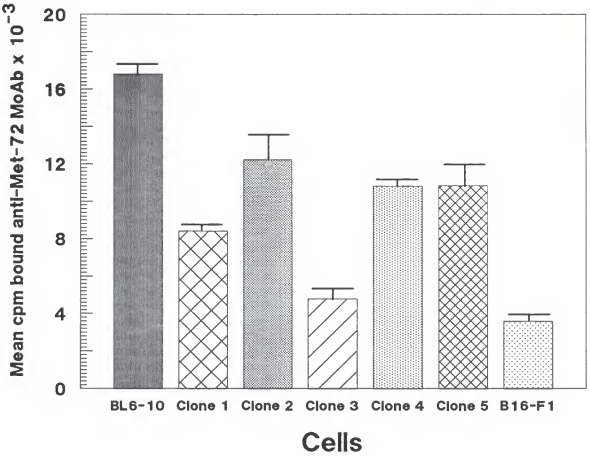

Mean cpm bound anti-Met-72 MoAb x 10^{-3}
20
16
12
8
4
0
BL6-10
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
B16-F1
Cells

Figure 7. Met-72 positive variants of B16 melanoma detected in cytospin and impression smear preparations of metastases.

Cell suspensions from a fresh ovarian metastasis were stained using biotinylated control N-S.7 MoAb (A) or anti-Met-72 MoAb (B). Impression smears of experimental lung metastases (C, D) or spontaneous lung metastases (E, F) were stained using biotin conjugated N-S.7 MoAb (C, E) or anti-Met-72 MoAb (D, F). (A - F x 270).

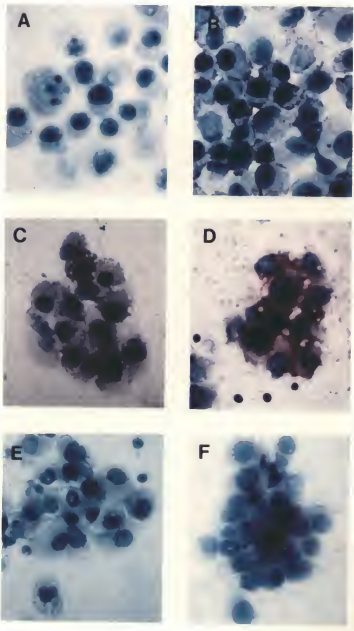

A
B
C
D
E
F

Figure 8. Localization of Met-72 positive variants within developing B16 subcutaneous melanoma.

Biotin conjugated N-S.7 MoAb bound to a cryostat section of a B16-F1 subcutaneous melanoma (A) x 55 and (B) x 135. Biotin conjugated anti-Met-72 MoAb bound to a serial section of the same B16-F1 s.c. melanoma x 55 (C), x 135 (D), x 1350 (E), x 550 (F).

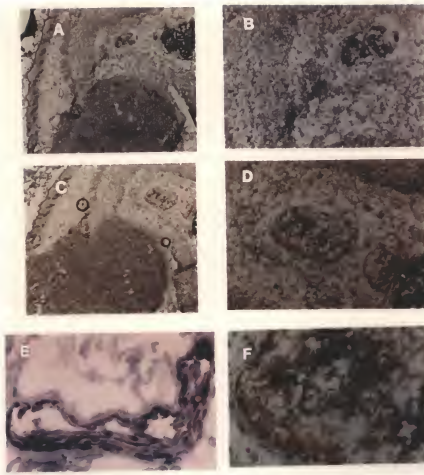

A
B
C
D
E
F

Figure 9. Direct immunofluorescence of Met-72 positive variants localized within B16 melanoma metastases.

Experimental B16-F10 lung (A - C) and B16-F1 liver (D - G), metastases were generated and frozen as described. Serial sections (8 μm) were cut and stained with FITC-conjugated N-S.7 MoAb (A and D x 270) or with FITC-conjugated anti-Met-72 MoAb (B and E x 270; C, F and G x 550).

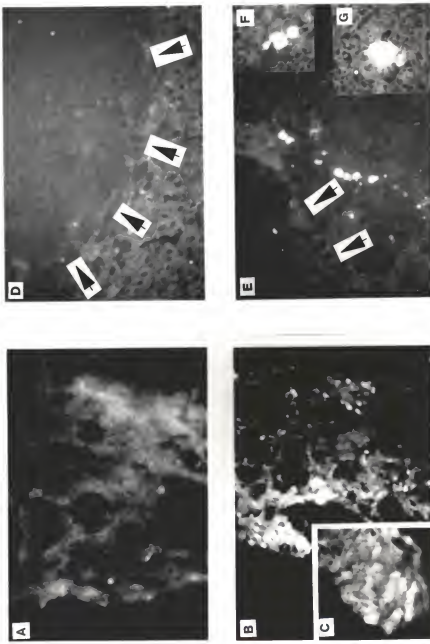

A
B
C
D
E
F
G

Table 2. Syngeneic Immune Sera Reactivity with C57BL/6 Melanomas

Female C57BL/6 mice were injected i.v. with 1, 2 or 4 X 10^5 B16-F1, BL6-10 or JB/RH cells. One or two animals per cell dose group were exsanguinated under gas anesthesia 8 days after inoculation. Individual sera at a final dilution of 1:50 were tested in duplicate by RIA for binding to B16-F1, BL6-10 and JB/RH cells harvested from culture. Littermate and age matched normal sera at a final dilution of 1:50 indicated background binding: mean ± S.D. bound to B16-F1 = 1434 ± 141; to BL6-10 = 422 ± 29; to JB/RH = 2189 ± 47. The RIA shown is one of two replicate experiments using the same immune serum. The generation of this immune response has been reproduced in independent replicate experiments six times.

Tumor Inoculum	Cell # X 10^5	Mean cpm Immune Sera Bound ± S.D. B16-F1	BL6-10	JB/RH
B16-F1	1	4461 ± 411	455 ± 1	7542 ± 317
	1	3594 ± 439	441 ± 49	9350 ± 0
	2	7160 ± 12	822 ± 11	12218 ± 1927
	2	7389 ± 485	302 ± 22	18295 ± 0
	4	8230 ± 36	604 ± 6	13479 ± 432
	4	7085 ± 434	603 ± 41	17212 ± 0
BL6-10	1	1437 ± 15	433 ± 66	1687 ± 137
	1	1510 ± 204	550 ± 25	1785 ± 177
	2	1531 ± 111	536 ± 0	1783 ± 62
	2	1687 ± 98	836 ± 4	2243 ± 158
	4	1529 ± 41	349 ± 30	1347 ± 87
	4	1432 ± 68	371 ± 66	1482 ± 163
JB/RH	1	5107 ± 342	417 ± 72	10579 ± 1143
	2	8762 ± 231	502 ± 13	16453 ± 2038
	4	12700 ± 722	599 ± 46	25949 ± 0

Table 3. Syngeneic Immune Sera Reactivity with C57BL/6 Melanomas

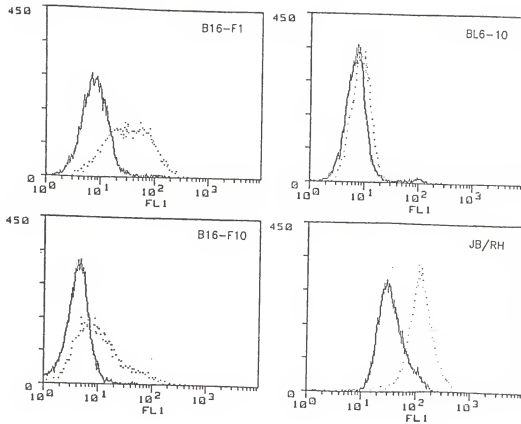
Female C57BL/6 mice were injected i.v. with 1, 2 or 4 X 10^5 B16-F1, BL6-10 or JB/RH cells. One or two animals per cell dose group were exsanguinated under gas anesthesia 16 days after inoculation. Individual sera at a final dilution of 1:50 were tested in duplicate by RIA for binding to B16-F1, BL6-10 and JB/RH cells harvested from culture. Littermate and age matched normal sera at a final dilution of 1:50 indicated background binding: mean ± S.D. bound to B16-F1 = 1535 ± 18; to BL6-10 = 662 ± 103; to JB/RH = 1770 ± 59. The RIA shown is one of two replicate experiments using the same immune serum. The generation of this immune response has been reproduced in independent replicate experiments six times.

Tumor Inoculum	Cell # X 10^5	Mean cpm Immune Sera Bound ± S.D. B16-F1	BL6-10	JB/RH
B16-F1	1	13372 ± 1353	521 ± 6	23617 ± 1945
	1	6798 ± 1173	354 ± 25	11662 ± 1476
	2	10174 ± 1884	438 ± 51	18379 ± 2408
	2	4891 ± 192	434 ± 12	8891 ± 453
	4	9275 ± 943	580 ± 133	19369 ± 11664
	4	11363 ± 871	628 ± 74	22635 ± 366
BL6-10	1	1398 ± 80	364 ± 7	1479 ± 72
	1	1315 ± 1	329 ± 1	1421 ± 83
	2	1464 ± 122	549 ± 4	1624 ± 50
	2	1529 ± 117	522 ± 3	1519 ± 25
	4	1281 ± 217	564 ± 33	1455 ± 180
	4	1452 ± 93	751 ± 4	1674 ± 16
JB/RH	1	22313 ± 1780	468 ± 37	39984 ± 2689
	2	23102 ± 1978	681 ± 26	41698 ± 7111

Figure 10. Flow cytometric analysis of anti-B16-F1 immune sera (A) and anti-B16-F1 MoAb (2D8.3B1) (B) binding to C57BL/6 melanomas.

The solid line indicates control Ab binding; the stippled line indicates anti-B16-F1 Ab binding. The antisera is from the same individual mouse (challenged i.v. with 3 X 10^5 B16-F1 cells) which was used to generate the anti-B16-F1 hybridoma, 2D8. Log green fluorescence is on the x axis; relative cell number per channel on the y axis.

A


450
B16-F1
0
10^0 10^1 10^2 10^3
FL1
450
BL6-10
0
10^0 10^1 10^2 10^3
FL1
450
B16-F10
0
10^0 10^1 10^2 10^3
FL1
450
JB/RH
0
10^0 10^1 10^2 10^3
FL1

B

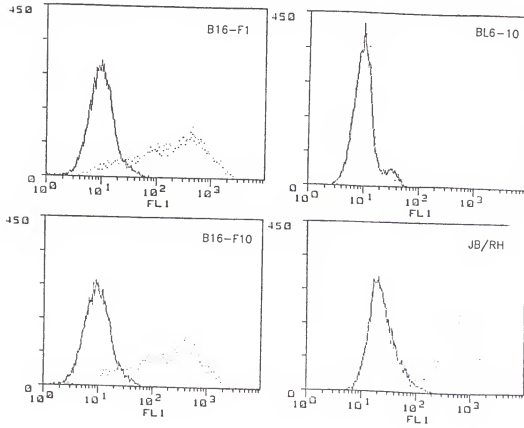
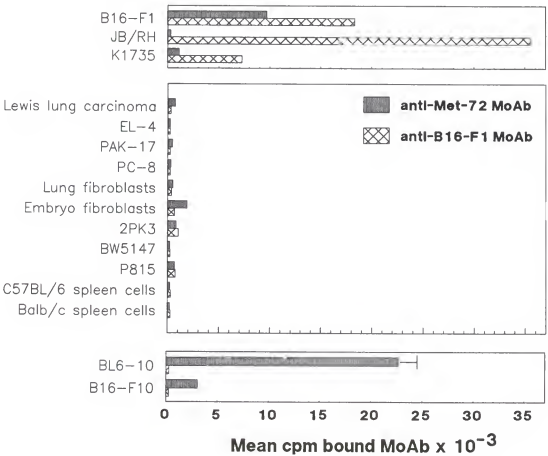

450
B16-F1
0
10^0 10^1 10^2 10^3
FL1
450
BL6-10
0
10^0 10^1 10^2 10^3
FL1
450
B16-F10
0
10^0 10^1 10^2 10^3
FL1
450
JB/RH
0
10^0 10^1 10^2 10^3
FL1

Figure 11. Anti-B16-F1 monoclonal antibody binding to a panel of murine cells.

Exhaustion supernates of anti-Met-72 or anti-B16-F1 (2D8.3B1) MoAb were reacted with cells harvested from culture or spleen cell suspensions. Antibody remaining bound after washes was indirectly indicated by RIA as described. Values are expressed as the mean of duplicate determinations of cpm bound ± S.D. All S.D. except one were less than 1%. Background binding of N-S.7 MoAb was routinely less than 1000 ± 10%. Data shown is representative of two experiments.

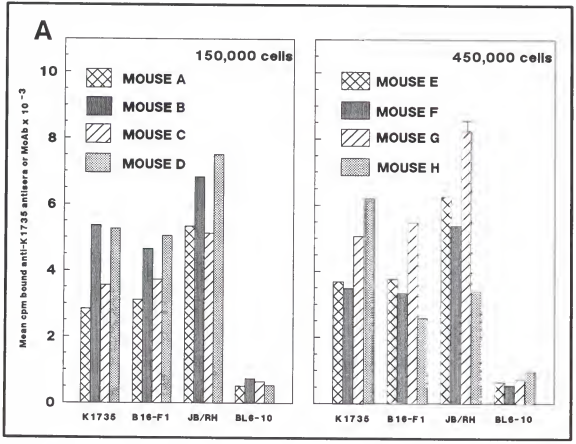


cell inoculum	* mice	* lung nodules mean	range
B16–F1	15	12	3–30
JB/RH	12	0	0

BL6–10	9	288	190–300
B16–F10	11	161	53–300

Figure 12. Syngeneic antibody response against K1735 melanoma.

C3H/HeJ mice were injected i.v. with 1.5 X 10^5 or 4.5 X 10^5 K1735 cells per 0.2 ml PBS. Sera was collected 21 days after inoculation and individual responses (numbered) were tested in RIA for binding against the syngeneic immunizing line and allogeneic melanoma metastatic variants (A). The cell lines were compared for binding to anti-Met-72 or anti-B16-F1 MoAb (2D8.3B1) in the same assays (B). Values are expressed as the mean of duplicate determinations of cpm bound ± S.D. All S.D. except one were less than 1%. Background binding of N-S.7 MoAb was routinely less that 800 ± 6%. Data shown is representative of two experiments.


A
150,000 cells
450,000 cells
MOUSE A
MOUSE B
MOUSE C
MOUSE D
MOUSE E
MOUSE F
MOUSE G
MOUSE H
Mean cpm bound anti-K1735 antisera or MoAb x 10 -3
10
8
6
4
2
0
K1735
B16-F1
JB/RH
BL6-10
K1735
B16-F1
JB/RH
BL6-10

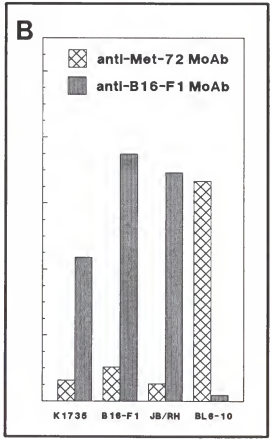

B
anti-Met-72 MoAb
anti-B16-F1 MoAb
K1735
B16-F1
JB/RH
BL6-10

Figure 13. Flow cytometric analysis of B16-F1 cells before and after immunomagnetic bead selection and removal of reactive subpopulations.

B16-F1 cells reacted against control (solid line) or immune Ab (stippled line) were analyzed by FACS after FITC-SAM binding. Subpopulation reactivity is shown in panels A (anti-B16-F1 antisera) and B (anti-B16-F1 MoAb, 2D8.3B1). Antibody labeled cells were removed by two cycles of immunomagnetic bead selection and restained using anti-B16-F1 antisera (C, F) or anti-B16-F1 MoAb (D, E). Log green fluorescence is on the x axis; relative cell number per channel (maximum = 350) on the y axis.

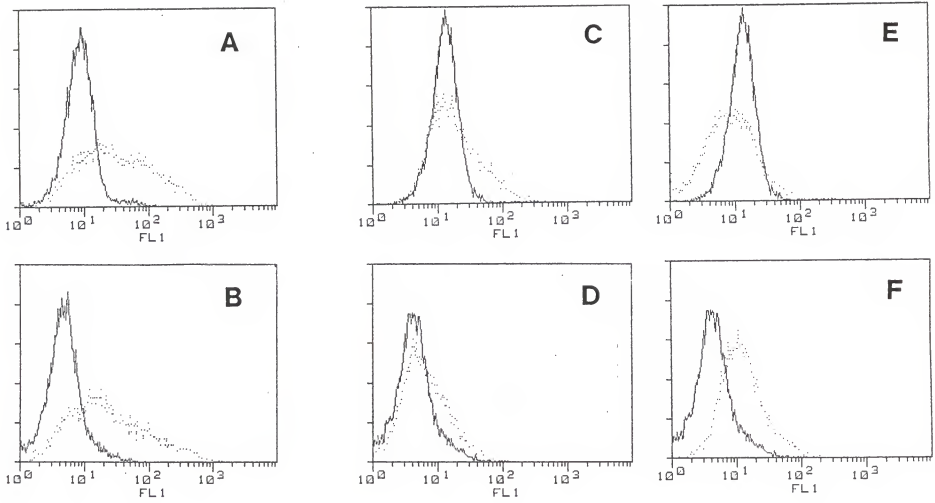
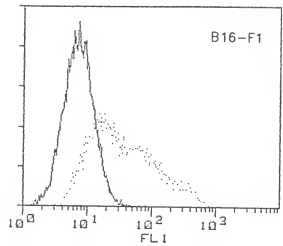
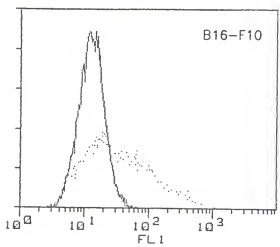

A
B
C
D
E
F
10^0
10^1
10^2
10^3
FL1

Figure 14. Flow cytometric analysis of B16 melanoma before and after immunomagnetic bead selection and removal of reactive subpopulations.

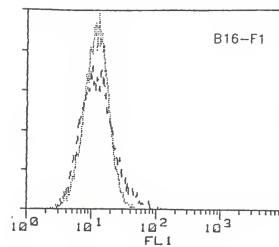
B16-F1 and B16-F10 cells reactive with NMS (solid line) or anti-B16-F1 antisera (stippled line) before and after immunomagnetic bead depletion were analyzed by FACS after FITC-SAM binding. Log green fluorescence is on the x axis; relative cell number per channel (maximum = 350) on the y axis.

BEFORE BEADING





AFTER BEADING



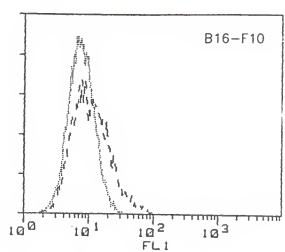
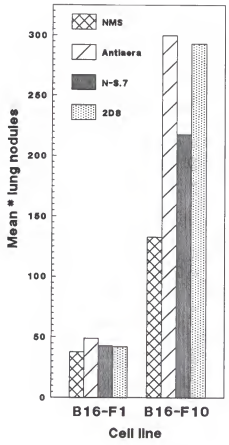


Figure 15. Experimental metastatic activity of negatively selected B16 melanoma cells.

B16-F1 and B16-F10 cells remaining after immunomagnetic bead depletion using NMS, anti-B16-F1 antisera, control N-S.7 MoAb or anti-B16-F1 MoAb (2D8.3B1) were injected i.v. (3×10^5 cells per 0.2 ml PBS) into 4 to 10 mice per group. Animals were necropsied 18 days later. The extrapulmonary metastatic tumor load within each animal was given a numerical score of severity for each organ site at necropsy (1 = least severe, 5 = most severe). The sum of the scores for each involved organ within an animal, was divided by the number of mice per group to obtain a mean (the index of extrapulmonary metastatic tumor load for a specific organ). The sum of the numerical scores for all involved organs included lymph nodes, ovaries, bone marrow, heart, adrenal gland, liver and uterus to equal the total index. Data shown has been repeated in an independent experiment using the same antisera and MoAb.


NMS
Antisera
N-S.7
2D8
Mean * lung nodules
0
50
100
150
200
250
300
B16-F1
B16-F10
Cell line

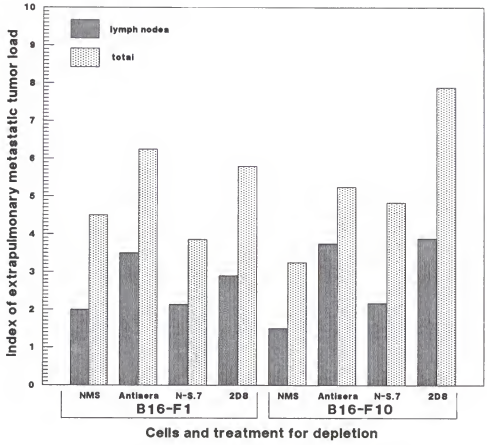

lymph nodes
total
Index of extrapulmonary metastatic tumor load
0
1
2
3
4
5
6
7
8
9
10
NMS
Antisera
N-S.7
2D8
B16-F1
NMS
Antisera
N-S.7
2D8
B16-F10
Cells and treatment for depletion

Table 4. Results of Analysis of Variance Comparing Groups of Mice Injected with Negatively Selected B16 Melanoma Cells

Cell Line	Treatment for Depletion	P Value Comparing Groups		
		mean # lung nodules	index of extrapulmonary tumor load lymph nodes	total
B16-F1	normal mouse serum versus anti-B16-F1 antisera	0.7204	0.0978	0.4720
B16-F10	normal mouse serum versus anti-B16-F1 antisera	0.0001	0.0196	0.4126
B16-F1	control N-S.7 MoAb versus anti-B16-F1 MoAb (2D8.3B1)	0.9696	0.1912	0.2283
B16-F10	control N-S.7 MoAb versus anti-B16-F1 MoAb (2D8.3B1)	0.0027	0.0103	0.0896

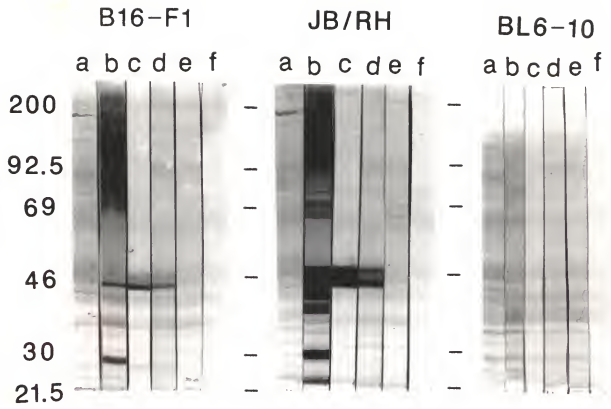


Figure 16. Western blot analysis of anti-B16-F1 immune sera and MoAb binding to lysates of syngeneic melanoma metastatic variant cell lines.

A 10% polyacrylamide gel was western blotted as described. Apparent molecular weights of marker proteins (Mr X 10^{-3}) are indicated on the abcissa.

a. Normal mouse serum.

b. Anti-B16-F1 antisera.

c. Anti-B16-F1 MoAb, 1F11.1E12.

d. Anti-B16-F1 MoAb, 2D8.3B1.

e. Anti-B16-F1 MoAb, 2H5.1B1.

f. Anti-sheep RBC MoAb, N-S.7.

Figure 17. Immunocytology of metastatic variants exfoliated from fresh experimental lymph node metastases of BL6-10.

Impression smears of expermental lymph node metastases of BL6-10 were processed by the ABC immunoperoxidase method as described. The level of staining generated by N-S.7 MoAB (A) is compared with anti-Met-72 MoAb (B) and anti-B16-F1 MoAb, 2D8.3B1 (C) (A, B and C x 1350).

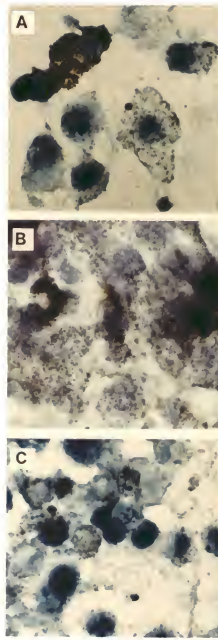

A
B
C

Figure 18. Localization of B16 melanoma metastatic variants within metastases by immunoperoxidase cytochemistry.

Serial cryostat sections (12 μm) of experimental metastases derived from B16-F10 were examined as described. Background (N-S.7) binding is seen in A compared with anti-Met-72 MoAb binding (B). (A and B x 1350).

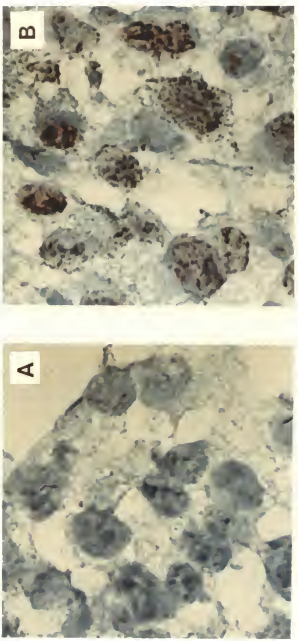

B
A

Figure 19. Localization of B16 melanoma variants within experimentally colonized lungs.

Mice were injected i.v. with 3 $X10^5$ B16-F1 cells per 0.2 ml PBS; colonized lungs were removed and snap frozen at necropsy 21 days later. Serial cryostat sections (12 μm) were analyzed by the ABC immunoperoxidase technique as described. Control (N-S.7) binding is shown in A and B; anti-Met-72 MoAb binding in C, D and G; and anti-B16-F1 MoAb (2D8.3B1) binding in E and F. (A, C and E x 135; B, D, F and G x 1350).

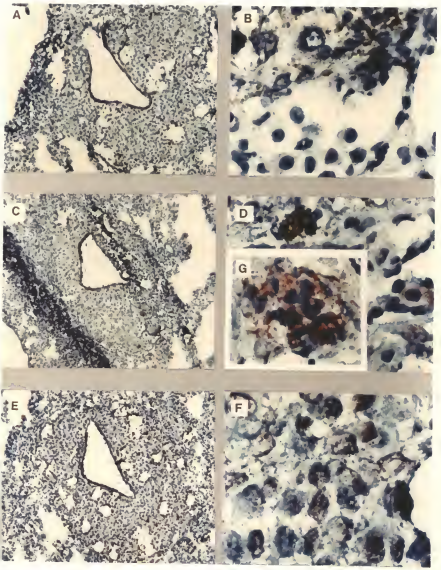

A
B
C
D
G
E
F

Table 5. Level of Monoclonal Antibody Binding to Cryostat Sections of Subcutaneous B16 Melanomas.[a]

Cell Line	Tumor Bearer[b]	Mean Tumor Volume (mm^3) ± S.D.	Level of Binding Against[c] anti-Met-72 MoAb	anti-B16-F1 MoAb
B16-F1	C57BL/6 normal	172 ± 11	+	-
	C57BL/6 500 Gy	518 ± 60	+ +	+
	Balb/c nu/nu	103 ± 18	+	+
BL6-10	C57BL/6 normal	202 ± 32	+ + +	-
	C57BL/6 500 Gy	735 ± 21	+ + + + +	+
	Balb/c nu/nu	120 ± 28	+ + + +	+

a. Subcutaneous tumors induced by injection of 2 $X10^6$ cells per 0.1 ml PBS were excised after 12 days and snap frozen. Serial sections (12 μm) were examined by ABC immunoperoxidase analysis as described.

b. Female mice 10 to 12 weeks old at the time of tumor excision were not treated (C57BL/6 normal), 500 Gy irradiated on the day of injection (C57BL/6 500 Gy), or were allogeneic athymic hosts (Balb/c nu/nu).

c. Biotin conjugated N-S.7 MoAb indicated negative or background levels of binding for comparison.

Figure 20. Localization of B16 melanoma variants within subcutaneous tumors maintained in normal syngeneic hosts.

A s.c. mass induced by injection of 2 X 10^6 BL6-10 cells per 0.1 ml PBS into a normal C57BL/6 mouse was excised after 12 days and snap frozen. Serial cryostat sections (12 μm) were examined by the ABC immunoperoxidase technique as described. Control N-S.7 MoAb binding is shown in A and B; anti-Met-72 MoAb in C and D; and anti-B16-F1 MoAb (2D8.3B1) in E and F. (A, C and E x 135; B, D and F x 1350).

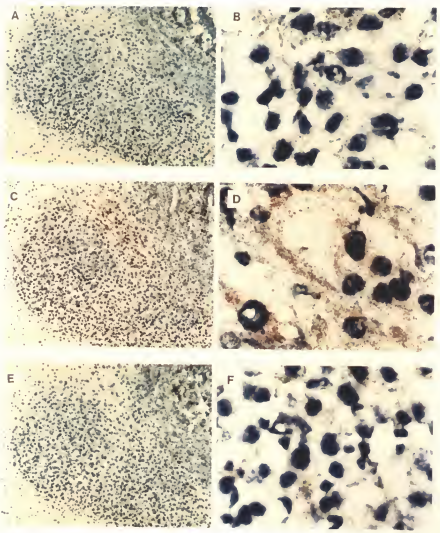

A
B
C
D
E
F

Figure 21. Localization of B16 melanoma variants within subcutaneous tumors maintained in 500 Gy irradiated syngeneic hosts.

A C57BL/6 mouse was exposed to 500 Gy irradiation and injected with 2 X 10^6 BL6-10 cells per 0.1 ml PBS on the same day. A s.c. mass was excised after 12 days, snap frozen and serial cryostat sections (12 μm) were examined by ABC immunoperoxidase analysis as described. Control N-S.7 MoAb binding is shown in A and B; anti-Met-72 MoAb in C, D and G; and anti-B16-F1 MoAb (2D8.3B1) in E and F. (A, C and E x 135; B, D, F and G x 1350).

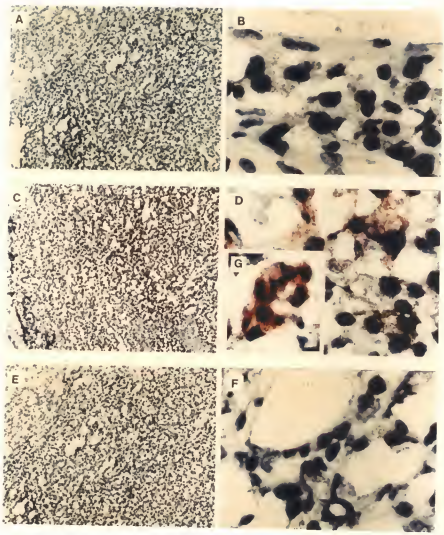
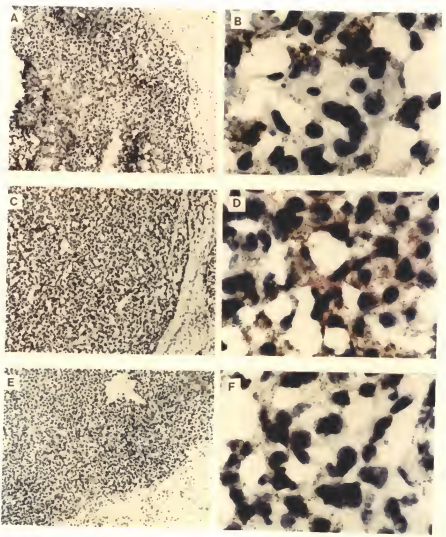

A
B
C
D
G
E
F

Figure 22. Localization of B16 melanoma variants within subcutaneous tumors maintained in allogeneic athymic hosts.

A s.c. mass induced by injection of 2 $X10^6$ BL6-10 cells per 0.1 ml PBS into a Balb/c nu/nu mouse was excised after 12 days and snap frozen. Serial cryostat sections (12 μm) were examined by the ABC immunoperoxidase technique as described. Control N-S.7 MoAb binding is shown in A and B; anti-Met-72 MoAb in C and D; and anti-B16-F1 MoAb (2D8.3B1) in E and F. (A, C and E x 135; B, D and F x 1350).


A
B
C
D
E
F

DISCUSSION

These studies have focused on 1) the localization of B16 melanoma metastatic variants within developing s.c. tumors and metastases and 2) the effect of the tumor bearer's humoral immune response on the regulation of the levels of metastatic variants within developing B16 melanomas.

The significant observations were first, that Met-72 expression is discretely localized in situ in the primary B16 melanoma mass. Met-72 positive variants are found to be variably melanotic cells regionally located within developing s.c. tumors grown in normal syngeneic hosts. Secondly, Met-72 expression is enriched on experimentally derived metastases. As increased ovarian colonizing potential is generated, Met-72 expression of melanoma cells derived from metastases to ovaries is enriched. There is individual cellular heterogeneity in the level of expression of Met-72, as visualized by immunocytology of metastases, with small foci of cells being the most positive within colonized organs. Thirdly, melanoma lines with poor experimental metastatic activity consistently elicit strong syngeneic Ab responses, while highly metastatic clonal variants do not. This Ab response can be used to subdivide metastatic from nonmetastatic cells present within the immunizing population.

Finally, the Ab response to poorly metastatic variants detects uniformly distributed variants within larger metastatic foci in experimentally colonized lungs.

The existence of subpopulations of cells exhibiting a range of metastatic potential within heterogeneous tumors has been substantiated in a number of systems (3,55). In the original studies leading to the present work, a strong correlation between the quantitative expression of a Mr 72,000 glycoprotein (Met-72) and experimental metastatic activity of over 30 in vitro grown B16 melanoma clones was demonstrated (5,11). Flow cytometric analysis and cell sorting procedures using anti-Met-72 MoAb have directly shown that high levels of Met-72 expression is characteristic of cells with a high experimental metastatic potential (12). Our current studies were designed to determine the potential utility of anti-Met-72 MoAb to visualize and localize Met-72 positive metastatic variants within progressing and metastatic B16 melanoma masses.

Results of the experiments reported here greatly expand our previous knowledge of Met-72 antigen expression and its correlation with metastatic potential in vivo. Localization of its expression in primary s.c. tumors is notably discrete, and not randomly distributed throughout the developing tumor mass (Figures 8 and 20) in immunologically normal syngeneic hosts. Immunohistologic examination of progressing tumors, excised at sequential times during s.c. growth, shows a

recurrent pattern of localization of Met-72 positive variants. These variably melanotic cells are seen within regions of the masses, the most brightly positive cells within smaller satellites. The implications of this selective localization are twofold. First, induction of Met-72 positive variants may be controlled by microenvironmental factors. Secondly, even if random somatic mutational events yield single metastatic variant cells within a solid tumor mass, their outgrowth into colonies may be directed by chemotactic factors which are microenvironmentally determined. These observations suggest that microenvironmental influences may function regionally to influence metastatic potential. Our observations in the B16 melanoma model of metastasis are consistent with those of Gabbert, et al. (56). They suggest that morphologic transitions at the invading front of rat malignant carcinoma may signify a localized process of tumor de-differentiation. Tumor cell locomotion may be specifically enhanced in regions observed to have a loss of basement membrane and decreased numbers of desmosomes between tumor cells. Clearly, the interactions in tumor microregions greatly influence tumor heterogeneity, as recently reviewed by Sutherland (57).

An important aspect of these studies focused on isolation and visualization of Met-72 positive variants within metastatic foci of B16 melanoma. The sophisticated capabilities of the fluorescence activated cell sorter have provided evidence that Met-72 expression may be a common

surface phenotype of B16 melanoma metastatic variants, irrespective of their organ colonization after i.v. inoculation. Experimentally induced ovarian metastases were directly shown to express Met-72 (54), as has been reported for experimental lung metastases of the B16 melanoma (12). As previously demonstrated with lung colonizing melanoma cells (58,59), ovarian colonizing variants were selected by repeated in vivo cycling. These variants showed increased levels of Met-72 positivity (Figure 5). Clones of bone marrow derived metastases also demonstrated increased levels of Met-72 expression (Figure 6). Clones derived from B16-F1 metastases to the liver, heart, lymph node and stomach wall have all demonstrated increased Met-72 antigen expression which is maintained after repeated in vitro passage.

Rapid visualization of individual cellular expression of Met-72 was achieved by two immunocytologic methodologies: 1) cytospin preparations of experimental ovarian metastases and 2) impression smears of experimental and spontaneous lung metastases. The ease of impression smear immunocytochemistry, especially, has permitted rapid characterization of the surface phenotype of cells dislodged from colonized lungs. Primary melanomas and other well encapsulated masses do not present suitable specimens for impression smears.

The significant findings of these studies are that (1) Met-72 expression is discretely localized in situ in the primary B16 melanoma mass, and (2) cell surface expression of

Met-72 may prove to be a generalized phenomenon of melanoma metastatic variant populations, regardless of organ selectivity. The study of metastatic tumor cell evolution influenced by 1) interactions with tumor derived elements and 2) host selection pressures during tumor progression has eluded tumor biologists. The ability to isolate metastatic variant cells from primary tumor tissue may enable us to quantitate their presence and evaluate their differences during tumor progression and metastatic outgrowth.

With few exceptions, humoral immunity towards syngeneic tumors has been generally regarded as beneficial for host survival (4,6,55,57,60). This belief, and the availability of therapeutic quantities of "tumor reactive" MoAb has resulted in a number of clinical trials for the treatment of human cancer.

The notion that poorly immunogenic variants exist within tumors has been supported primarily by the isolation of subpopulations of tumor cells which are resistant to cellular effector systems in vitro (61-64). A major question left unanswered by these studies is the extent to which humoral immunity may contribute to the changing proportion of metastatic variants within tumor populations. Previous studies have not attempted to correlate syngeneic Ab responses against B16 melanoma-associated antigens with spontaneous or experimental metastatic activity of melanoma subpopulations (14-16,20,65,66). Some studies have indirectly correlated

syngeneic anti-B16 melanoma responses with metastatic activity (13,17,18) or have found no correlation between metastatic potential of clones derived from B16 melanoma and syngeneic Ab responses induced by s.c. immunization with those clones (19). McDonald, et al. (67) reported syngeneic Ab detected in sera of mice challenged with the highly invasive variant of B16 melanoma, B16-BL6. However the subpopulation and functional specificity of these Ab was not determined.

The ease and frequency with which host anti-melanoma humoral immunity can be demonstrated in both experimental and clinical situations has prompted us to examine whether Ab responses in these situations may in fact be targeted against functionally distinct subsets of melanoma. While studies directly correlating metastatic activity and immunogenicity of melanoma subpopulations have not been described previously, several lines of investigation have suggested the possibility that poorly metastatic subpopulations are the primary Ab targets. First, syngeneic and autochthonous Ab responses against melanoma are frequently observed (13-20,65-67). However, detection of syngeneic Ab reagents defining highly metastatic melanoma variants often require manipulation of host immunity to override these primary responses (5). Second, Ab against human melanoma, obtained by xenogeneic immunization, are most reactive with the more differentiated, benign melanoma forms (68-71). Recently, Herd reported the production and characteristics of MoAb from syngeneic mice

bearing B16 melanoma masses (72). Some of the MoAb were reported to enhance or inhibit B16-F1 lung colonization and at the same time to decrease lung colonization of the high lung colonizing variant, B16-F10. Recent studies by others within our laboratory now suggest that in addition to the primary host responses against subpopulations of poorly metastatic B16 melanoma, the isotype of these antibodies has a dramatic biologic consequence (Kimura et al., unpublished results). Mice challenged i.v. with syngeneic B16-F1 or BL6-10 cells after i.p. treatment with the IgG_3 isotype switch variant of anti-Met-72 MoAb were observed to have increased numbers of lung nodules.

While it is true that syngeneic anti-melanoma MoAbs which interfere with experimental metastasis can be isolated (5,65,72), these Ab responses are rare. In these cases multiple immunizations, with or without adjuvant, coupled to highly specific screening procedures are needed to isolate these reactivities. The major finding to emerge from our study is that melanoma lines with poor metastatic activity consistently elicit strong syngeneic Ab responses, while highly metastatic clonal variants do not. These anti-nonmetastatic Ab activities are readily demonstrated by analyzing the serum from melanoma immunized mice. Poorly metastatic melanoma lines such as B16-F1 and JB/RH elicit Ab responses which can be used to further subdivide metastatic from non-metastatic cells present within the immunizing

population. This conclusion is based on 1) Ab binding to well characterized melanoma lines and clones shown by RIA, FACS, and western blot analysis, and 2) experimental metastatic capabilities of negatively selected cells.

The results of our immunomagnetic bead depletion assays show a total increase in the extent of experimental metastases generated after the removal of anti-B16-F1 reactive subpopulations. This increase is subtle with the heterogeneous parental B16-F1 line, but significant with the B16-F10 line. It is not unexpected that a subpopulation of anti-B16-F1 antisera reactive cells remains after removal of the anti-B16-F1 MoAb reactive cells (Figure 13 F). Our results support the suggestion that B16-F10 was selected for increased lung colonization potential, but that organ selectivity is not absolutely equivalent to increased metastatic potential. B16-F1 and B16-F10 are heterogeneous lines with respect to many phenotypes (6). Metastases generated from B16-F1 and B16-F10 may maintain heterogeneity with regard to organ colonization potential. Our findings are consistent with the interpretation that non-metastatic cells present within the parental B16-F1 line (and at lower levels in the selected B16-F10 line) elicit a strong and selective Ab response; the negative binding cells representing the more highly metastatic variants. Removal of the poorly metastatic variants from the total population allows the extrapulmonary colonization potential to become more evident. This is more

so with B16-F10, because on a cell to cell basis this heterogeneous population contains more cells with a more efficient metastatic potential.

The mechanism of this extrapulmonary colonization may be suggested by the results of experiments by Nakajima, et al. (73). Metastatic variants of B16 melanoma, including B16-F1 and B16-F10, were shown to differ from each other in their ability to degrade heparan sulfate which may be related to their ability to adhere and grow in specific organs (73). Specificity of adhesion between murine tumor cells and microvascular endothelium from different organs has been demonstrated by Auerbach et al. (74). Thus, variants remaining after depletion of anti-B16-F1 reactive subpopulations may mainfest their adhesive phenotypes more efficiently.

The influence of host immunity on the induction or dissemination of metastatic variants appears variable depending upon which of the heterogeneous phenotypes required for tumor progression one is attempting to study (1-4). The studies reported here strongly suggest the relevance of comparing metastatic variant clonal lines with heterogeneous lines. Caution must be exercised when interpreting results obtained using heterogeneous lines which appear homogeneous with regard to one phenotype, such as B16-F10 cells.

Anti-B16-F1 antisera and MoAb have recently been used to immunoprecipitate from detergent lysates of melanoma cells

biosynthetically labeled with [^{3}H]-leucine, bands which correspond to those identified by western blot analyses (Mr 32,000, 45,000 and 55,000 on 5 to 15% or 10% acrylamide slab gels under reducing conditions, Parratto & Kimura, unpublished results). Molecular weight characteristics of anti-B16-F1 reactive species bear some resemblance to those reported by others (14-16,20,75-79). However, surface expression of those markers versus metastatic behavior of the cells was not determined in those studies. The Mr 45,000 band identified by the anti-B16-F1 antisera or corresponding MoAb cannot be against normal Class I antigens of the H-2 complex, as there is no measurable binding to C57BL/6 spleen cells or other syngeneic tumor cell lines.

Additional immunohistologic analyses of cells isolated from and localized within developing B16 melanoma metastases revealed several interesting binding profiles. First, impression smears revealed an increased level of anti-Met-72 MoAb binding to individual cells exfoliated from the parietal lung surface compared to cells exfoliated from cut surfaces of well encapsulated extrapulmonary metastases. On the other hand, anti-B16-F1 MoAb binding was weak to cells exfoliated from these same metastases. In addition, cryostat sections of colonized organs showed intensely Met-72 positive variants to be variably melanotic individual cells or cells within small metastatic foci. However, anti-B16-F1 MoAb showed a diffuse pattern of reactivity with melanotic cells within larger B16-

F1 pulmonary metastases and moderate binding to extrapulmonary metastases. A morphologic assessment of B16 metastases within the syngeneic host has been reported recently by Dingemans (80). Patterns of metastatic growth within experimentally colonized liver and lung were compared revealing small, dense tumor cell nodules within encapsulated spheres (liver) or flat, ill defined foci (lung). This was interpreted as apparent regions of focal proliferation alternating with active movement and was explained on the basis of interactions with extracellular matrix. Our studies show that the Met-72 positive variants are observed within microscopi foci of colonized organs. These observations suggest that Met-72 antigen expression may be correlated to cells within regions of focal proliferation.

Our results localizing B16 melanoma variants within developing s.c. masses and metastases are in accordance with the observations of Yamasaki, et al. (81). Distinctly different regional distributions of radiolabeled syngeneic MoAb directed against B16 melanoma was observed in vivo in B16 melanoma masses. These patterns of distribution were attributed to differentiation stages of melanoma cells, however, MoAb binding was not correlated with metastatic potential in their study.

Analysis of metastatic variant localization within s.c. tumors maintained in syngeneic, sublethally irradiated or allogeneic, athymic hosts revealed a striking observation.

The number of Met-72 positive variants and their individual level of Met-72 expression was increased in s.c. masses maintained in irradiated or immunocompromised hosts. This observation may contradict the contention that the host Ab response is directed against poorly metastatic variants. Clearly these preliminary experiments warrant further investigation.

The basic biologic and practical implications of these findings suggest that host humoral immunity to melanoma may represent a major driving force favoring tumor progression and metastasis. Non-immunogenic subpopulations of melanoma of higher metastatic potential could thus continue to emerge and become the dominant cell type in the absence of other selective forces. Thus, anti-tumor MoAb raised by similar immunization protocols for therapeutic studies should first be used to examine the metastatic competence of the target population. Additional criteria besides "melanoma-specific" may be required and warranted, before passive immunotherapy involving MoAb is applied to clinical situations. Additional studies involving host humoral immunity and tumor progression are clearly warranted.

REFERENCES

1. Nowell, P. C. Mechanisms of tumor progression. Cancer Res., 46:2203-2207, 1986.

2. Nicolson, G. L. Tumor cell instability, diversification, and progression to the metastatic phenotype: From oncogene to oncofetal expression. Cancer Res., 47:1473-1487, 1987.

3. Nicolson, G. L., and Poste, G. Tumor cell diversity and host responses in cancer metastasis. Curr. Probl. Cancer, 6:4-83, 1982.

4. Nicolson, G. L. and Poste, G. Tumor cell diversity and host responses in cancer metastasis. Host immune responses and therapy of metastases. Curr. Probl. Cancer, 7:1-43, 1983.

5. Kimura, A. K. and Xiang, J. High levels of Met-72 antigen expression: Correlation with metastatic activity of B16 melanoma tumor cell variants. J. Natl. Cancer Inst., 76:1247-1253, 1986.

6. Fidler, I. J., and Nicolson, G. L. Immunobiology of experimental metastatic melanoma. Cancer Biol. Rev., 2:1-47, 1981.

7. Poste, G. Regulation of metastatic cellular subpopulations in malignant tumors. J. Cell. Biochem., Suppl 11D: 78, 1987.

8. Paulus, G., Hong, L. X., Atassi, G., and Buyssens, N. Regional variations in proliferaton rate and degree of differentiation in B16 melanomas. Virchow Arch. Cell. Pathol., 46:43-52, 1984.

9. Xiang, J., Kimura, A. K., and Hansen, J. P. Synthesis and expression of metastasis-associated, Met-72/83 antigens. Clin. & Exptl. Met., 6:473-483, 1988.

10. Kimura, A. K., Mehta, P., Xiang, J., Lawson, D., Dugger, D., Kao, K-J., and Lee-Ambrose, L. The lack of correlation between experimental metastatic potential and platelet aggregating activity of B16 melanoma clones viewed in relation to tumor cell heterogeneity. Clin. & Exptl. Met., 5:125-133, 1987.

11. Xiang, J. and Kimura, A. K. In vitro modulation of the metastatic phenotype. I. Analysis of differentiation forms of the B16 melanoma expressing Met-72 determinants and metastatic activity. Clin. & Exptl. Met., 4:293-309, 1986.

12. Xiang, J. and Kimura, A. K. Isolation of metastatic B16 melanoma variants using anti-Met-72 monoclonal antibodies and flow cytometry. Clin. & Expl. Met., 5:35-42, 1987.

13. Baniyash, M., Smorodinsky, N. I., Yaakubovicz, M., and Witz, I. P. Serologically detectable MHC and tumor-associated antigens on B16 melanoma variants and humoral immunity in mice bearing these tumors. J. Immunol., 129:1318-1323, 1982.

14. Poskitt, P. K. F., Poskitt, T. R., and Wallace, J. H. Release into culture medium of membrane-associated, tumor-specific antigen by B16 melanoma cells. Proc. Soc. Exptl. Biol. Med., 152:76-80, 1976.

15. Bystryn, J-C., Schenkein, I., Baur, S., and Uhr, J. W. Partial isolation and characterization of antigens associated with murine melanoma. J. Natl. Cancer Inst., 52:1263-1269, 1974.

16. Bystryn, J-C. Antibody response and tumor growth in syngeneic mice immunized to partially purified B16 melanoma associated antigens. J. Immunol., 120:96-101, 1978.

17. Johnston, D., Schachne, J. P., and Bystryn, J-C. Identification of immunogenic B16 melanoma-associated antigens. J. Biol. Resp. Mod., 6:108-120, 1982.

18. Stackpole, C. W., and Demsey A. Multiple antigens related to the major envelope glycoprotein of murine leukemia virus expressed on B16 melanoma cells as targets of host immune response. Invas. Metastasis, 4:28-46, 1984.

19. Stackpole, C. W., Alterman, A. L., Braverman, S., and Rappaport, I. Development of host immunity to phenotypically diverse B16 melanoma clones. Invas. Metastasis, 7:346-366, 1987.

20. Rappaport, I., Alterman, A. L., Braverman, S., and Stackpole, C. W. Syngeneic monoclonal antibodies to B16 melanoma viral antigens. Cancer Res., 47:5391-5396, 1987.

21. Real, F. X., Mattes, M. J., Houghton, A. N., Oettgen, H. F., Lloyd, K. O., and Old L. J. Class 1 (unique) tumor antigens of human melanoma. Identification of a 90,000 dalton cell surface glycoprotein by autologous antibody. J. Exptl. Med., 160:1219-1233, 1984.

22. Livingston, P. O., Kaelin, K., Pinsky, C. M., Oettgen, H. F., and Old, L. J. The serologic response of patients with stage II melanoma to allogeneic melanoma cell vaccines. Cancer, 56:2194-2200, 1985.

23. Wong, J. H., Gupta, R. K., and Morton, D. L. Demonstration of a well-characterized tumor-associated antigen on melanoma cell surface. J. Surg. Oncol., 38:147-150, 1988.

24. Vlock, D. R., Scalise, D., Meglin, N., Kirkwood, J. M., and Ballou, B. Isolation and partial characterization of melanoma-associated antigens identified by autologous antibody. J. Clin. Invest. 81:1746-1751, 1988.

25. Berkelhammer, J., Oxenhandler, R. W., Hook, R. R., and Hennessy, J. M. Development of a new melanoma model in C57BL/6 mice. Cancer Res., 42:3157-3163, 1982.

26. Berkelhammer, J., Luethens, T. N., Hook, R. R., and Oxenhandler, R. W. Phenotypic instability of mouse melanomas after propagation *in vivo* and *in vitro*. Cancer Res., 46:2923-2928, 1986.

27. Poste, G., Doll, J., Hart, I. R., and Fidler, I. J. *In vitro* selection of murine B16 melanoma variants with enhanced tissue-invasive properties. Cancer Res., 40:1636-1644, 1980.

28. Noonan, K. Analysis of concanavalin A mediated agglutination in two Chinese hamster ovary subclones whose surface phenotype respond to maintenance in medium supplemented with dibutyryl cyclic AMP. V. Biochemical composition of the plasma membrane. Biochim. Biophys. Acta, 551:22-43, 1979.

29. Click, R. E., Benck, L., and Alter, B. J. Immune response *in vitro*. I. Culture conditions for antibody synthesis. Cell. Immunology, 3:264-276, 1972.

30. Kripke, M. L. Speculations on the role of ultraviolet radiation in the development of malignant melanoma. J. Nat. Can. Inst., 63:541-548, 1979.

31. Parratto, N. P., Odebralski, J. M., and Kimura, A. K. Syngeneic antibody responses against murine melanoma are directed against poorly metastatic tumor cell variants. Cancer Research, submitted, 1988.

32. Ey, P. L., Prowse, S. J., and Jenkin, C. R. Isolation of pure IgG_1, IgG_{2a} and IgG_{2b} immunoglobulins from mouse serum using Protein A-Sepharose. Immunochem., 15:429-436, 1978.

33. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227:680-685, 1970.

34. Guesdon, J-L., Ternynck, T., and Avrameas, S. The use of avidin-biotin interaction in immunoenzymatic techniques. J. Histochem. Cytochem., 27:1131-1139, 1979.

35. Braylan, R. C., Benson, N. A., Nourse, V., and Kruth, H. S. Correlated analysis of cellular DNA, membrane antigens and light scatter of human lymphoid cells. Cytometry, 2:337-343, 1982.

36. Goding, J. W. Monoclonal antibodies: Principles and Practice. Academic Press Inc., New York. pp 223-233, 1983.

37. Pretlow, T. P., Stinson, A. J., Pretlow, T. G., and Glover, G. L. Cytologic appearance of cells dissociated from rat colon and their separation by isokinetic and isopyknic sedimentation in gradients of Ficoll. J. Nat. Can. Inst. 61:1431-1437, 1978.

38. Yam, L. T., English, M. C., Janckila, A. J., Zeismer, S., and Li, C-Y. Immunocytochemical characterization of human blood cells. Am. J. Clin. Path., 80:314-321, 1983.

39. Nakano, G. M., Natale, R. B., Lobuglio, A. F., and Houghton, A. N. Immunoperoxidase staining of early human melanoma colonies with monoclonal antibodies. Am. J. Pathol., 114:380-366, 1984.

40. Nuti, M., Mottolese, M., Viora, M., Donnorso, R. P., Schlom, J., and Natali, P. G. Use of monoclonal antibodies to human breast-tumor-associated antigens in fine-needle aspirate cytology. Int. J. Cancer, 37:493-498, 1986.

41. Perry, M. D., Seigler, H. F., and Johnston, W. W. Diagnosis of metastatic malignant melanoma by fine needle aspiration biopsy: A clinical and pathologic correlation of 298 cases. J. Nat. Can. Inst. 77:1013-1021, 1986.

42. Hsu, S-M., Raine, L., and Fanger, H. A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am. J. Clin. Pathol. 75:734-738, 1981.

43. Hsu, S-M., Raine, L., and Fanger, H. The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase technics. Am. J. Clin. Pathol. 75:816-821, 1981.

44. McConahey, P. J. and Dixon, F. J. A method of trace iodination of proteins for immunologic studies. Int. Arch. Allergy Appl. Immunol., 29:185-189, 1966.

45. Dorval, G., Welsh, K. I., and Wigzell, H. A. Radioimmunoassay of cellular surface antigens of living cells using iodinated soluble protein A from Staphylococcus aureus. J. Immunol. Methods, 7:237-249, 1975.

46. Philpott, G. W., Kulczycki, A., Grass, E. H., and Parker, C. W. Selective binding and cytotoxicity of rat basophilic leukemia cells (RBL-1) with immunoglobulin E-biotin and avidin-glucose oxidase conjugates. J. Immunol. 125:1201-1209, 1980.

47. Kimura, A. K., and Peck, A. B. A novel anti-Ia monoclonal antibody which specifically enhances the corresponding T cell alloreactivity. J. Immunol., 133:2360-2364, 1984.

48. Cruikshank, W. W., Berman, J. S., Theodore, A. C., Bernardo, J., and Center, D. M. Lymphokine activation of T4+ T lymphocytes and monocytes. J. Immunol., 138:3817-3823, 1987.

49. Kessler, S. Cell membrane antigen isolation with the staphylococcal protein A-antibody adsorbent. J. Immunol., 117:1482-1490, 1976.

50. Kessler, S. W. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: Parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol., 115:1617-1624, 1975.

51. Pluskal, M. G., Przekop, M. B., Kavonian, M. R., Vecoli, C., and Hicks, D. A. Immobilon PVDF transfer membrane: A new membrane substrate for Western blotting of proteins. Biotech., 4:272-283, 1986.

52. Towbin, H., Staehelin, T., and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci., 76:4350-4354, 1979.

53. Fidler, I. J., Gruys, E., Cifone, M. A., Barnes, Z., and Bucana, C. Demonstration of multiple phenotypic diversity in a murine melanoma of recent origin. J. Natl. Can. Inst., 67:947-956, 1981.

54. Parratto, N. P. and Kimura, A. K. Isolation and visualization of Met-72 positive, metastatic variants present in B16 melanoma tumor masses. J. Cell. Biochem., 36:311-322, 1988.

55. Welch, D. R. and Tomasovic, S. P. Implications of tumor progression on clinical oncology. Clin. & Exptl. Met., 3:151-188, 1985.

56. Gabbert, H., Wagner, R., Moll, R., and Gerharz, C-D. Tumor dedifferentiation: An important step in tumor invasion. Clin. & Exptl. Met., 3:257-279, 1985.

57. Sutherland, R. M. Cell and environment interactions in tumor microregions: The multicell spheroid model. Science, 240:177-184, 1988.

58. Fidler, I. J. and Nicolson, G. L. Organ selectivity for survival and growth of B16 melanoma variant tumor lines. J. Natl. Can. Inst., 57:1199-1202, 1976.

59. Poste, G., Doll, J., Brown, A. E., Tzeng, J., and Zeidman, I. Comparison of the metastatic properties of B16 melanoma clones isolated from cultured cell lines, subcutaneous tumors and individual lung metastases. Cancer Res., 42:2770-2778, 1982.

60. Alexander, P., and Eccles, S. A. Host-mediated mechanisms in the elimination of circulating cancer cells. In: Garth L. Nicolson and Luka Milas (eds.), Cancer Invasion and Metastasis: Biologic and Therapeutic Aspects, pp. 293-308. New York: Raven Press, 1984.

61. Fidler, I. J., Gersten, D. M., and Budmen, M. B. Characterization in vivo and in vitro of tumor cells selected for resistance to syngeneic lymphocyte-mediated cytotoxicity. Cancer Res., 36:3160-3165, 1976.

62. Fidler, I. J., Gersten, D. M., and Kripke, M. L. Influence of immune status on the metastasis of three murine fibrosarcomas of different immunogenicities. Cancer Res., 39:3816-3821, 1979.

63. Gorelik, E., Foget, M., Feldman, M., and Segal, S. Differences in resistance of metastatic tumor cells and cells from local tumor growth to cytotoxicity of natural killer cells. J. Natl. Cancer Inst., 63:1397-1404, 1979.

64. Eccles, S. A., and Alexander, P. Macrophage content of tumours in relation to metastatic spread and host immune reaction. Nature, 250:667-669, 1974.

65. Vollmers, H. P., and Birchmeier, W. Monoclonal antibodies inhibit the adhesion of mouse B16 melanoma cells in vitro and block lung metastasis in vivo. Proc. Natl. Acad. Sci., 80:3729-3733, 1983.

66. Vollmers, H. P., Imhof, B. A., Weiland, I., Hiesel, A., and Birchmeier, W. Monoclonal antibodies NORM-1 and NORM-2 induce more normal behavior of tumor cells in vitro and reduce tumor growth in vivo. Cell, 40:547-557, 1985.

67. McDonald, T. L., Collins, M., and Talmadge, J. E. Comparison of antibody isotypes in sera and circulating immune complexes during rumor growth and metastasis of three tumor models in mice. Cancer Res., 44:4933-4937, 1984.

68. Suter, L., Brüggen, J., Bröcker, E. B., and Sörg, C. A. A tumor-associated antigen expressed in melanoma cells with lower malignant potential. Int. J. Cancer, 35:787-791, 1985.

69. A. Houghton, A. N., Cordon-Cardo, C., and Eisenger, M. Differentiation antigens of melanoma and melanocytes. Int. Rev. Exptl. Path., 28:217-248. 1986.

70. Real, F. X., Houghton, A. N., Albino, A. P., Cordon-Cardo, C., Melamed, M. R., Oettgen, H. F., and Old, L. J. Surface antigens of melanomas and melanocytes defined by mouse monoclonal antibodies: Specificity analysis and comparison of antigen expression in cultured cells and tissues. Cancer Res., 45:4401-4411, 1985.

71. Herlyn, M. and Koprowski, H. Melanoma antigens: Biological characterization and clinical significance. Ann. Rev. Immunol., 6:283-308, 1988.

72. Herd, Z. L. Suppression of B16 melanoma lung colonization by syngeneic monoclonal antibodies. Cancer Res., 47:2696-2703, 1987.

73. Nakajima, M., Irimura, T., DiFerrante, D., DiFerrante, N., and Nicolson, G. L. Heparan sulfate degradation: Relation to tumor invasive and metastatic properties of mouse B16 melanoma sublines. Science, 220:611-613, 1983.

74. Auerbach, R., Lu, W. C., Pardon, E., Gumkowski, F., Kaminska, G., and Kaminski, M. Specificity of adhesion between murine tumor cells and capillary endothelium: An *in vitro* correlate of preferential metastasis *in vivo*. Cancer Res., 47:1492-1496, 1987.

75. Gersten, D. M. and Marchalonis, J. J. Demonstration and isolation of murine melanoma-associated antigenic surface proteins. Biochem. Biophys. Res. Comm., 90:1015-1024, 1979.

76. Bhavanandan, V. P., Kemper, J. G., and Bystryn, J-C. Purification and partial characterization of a murine melanoma-associated antigen. J. Biol. Chem., 255:5145-5153, 1980.

77. Nishio, C., Ishii, Y., Ishii, K., and Kikuchi, K. Identification of an oncofetal antigen (gp90) on murine B16 melanoma cells. Eur. J. Cancer Oncol., 18:579-588, 1982.

78. Khan, A., Grammer, S., Chang, D., Hill, N. O., and Miller, J. Mouse monoclonal antibody (WI-MN-I) against malignant melanoma. Cancer Res., 43:5868-5872, 1983.

79. Tomita, Y., Montague, P. M., and Hearing, V. J. Monoclonal antibody production to a B16 melanoma associated antigen. Int. J. Cancer, 35:543-547, 1985.

80. Dingemans, K. P. B16 Metastases in mouse liver and lung II. Morphology. Invas. Metastasis, 8:87-102, 1988.

81. Yamasaki, T., Wakabayashi, S., Inoue, O., Ando., K., Kusakabe, K., Kawasaki, Y., Okamoto, S., and Taniguchi, M. Specific biodetection of B16 mouse melanoma *in vivo* by syngeneic monoclonal antibody. J. Invest. Dermatol., **89**:225-229, 1987.

BIOGRAPHICAL SKETCH

I was born on April 24, 1956, in Philadelphia, Pennsylvania. My elementary education was in the public schools of Philadelphia; secondary education in Upper Dublin Township, Pennsylvania. I received a B.S. in biology from Villanova University, Villanova, Pennsylvania, May, 1978, and my D.V.M. from the University of Florida, Gainesville, Florida, May, 1985. My professional work experience has been in the area of veterinary medicine as an assistant from May, 1975, to December, 1978 with private practitioners in Pennsylvania. I hold a current license to practice veterinary medicine in Florida and have been a part time associate or relief veterinarian from August, 1985, to the present with private practitioners in Florida. My graduate research experience has been focused in the area of tumor immunology and cancer biology. As a graduate research assistant in the Department of Pathology, College of Medicine, University of Florida, I have trained with Dr. Richard T. Smith and Dr. Byron Croker. My dissertation mentor has been Dr. Arthur K. Kimura. I was awarded an NIH Traineeship in the Immunobiology of Cancer from the Fall of 1987 through the Fall of 1988; am named in Who's Who in Veterinary Science and Medicine, First Edition, 1987-88; and have received awards for service from

the President of the University of Florida, 1985, the Student Chapter of the American Veterinary Medical Association, 1985, and the American Veterinary Medical Association Auxiliary, 1984. I am a current member of the American Veterinary Medical Association and the American Association for the Advancement of Science. The research embodied in my dissertation has been presented as abstracts to three national scientific meetings and has been published and submitted for publication in refereed journals. I was offered post-doctoral fellowships for further training in cancer biology at Mount Sinai Research Institute, Toronto, Ontario, Canada; Scripps Clinic and Research Institute, La Jolla, California; the Medical College of Virginia, Massey Cancer Center, Richmond, Virginia; and the Department of Dermatology, New York University Medical School. I have accepted a position as staff scientist with a biotechnology corporation in Charleston, South Carolina, and as Adjunct Assistant Professor, Department of Laboratory Medicine and Immunology, Medical University of South Carolina.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Arthur K. Kimura, Chair
Associate Professor, Pathology
and Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Richard T. Smith
Professor, Pathology
and Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

K. Kendall Pierson
Professor, Pathology
and Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Sigurd Normann
Professor, Pathology
and Laboratory Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Maron B. Calderwood-Mays
Associate Professor, Pathology
and Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Chris West
Associate Professor, Anatomy and Cell Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1988

Dean, College of Medicine

Dean, Graduate School